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STUDY OF CARCINOGENIC MECHANISM BASED ON EXPERIMENTS WITH 4-NITROQUINOLINE N-OXIDE

(Plate I)

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INTRODUCTION

The mechanism through which normal cells undergo cancerous transformation cannot be elucidated by studying the origin of spontaneous cancers, which do not permit satisfactory experimental approaches. Discovery of many pure chemical carcinogens seemed to promise much, but, actually it now appears that they are so complex in their structures and reactions that the mechanism proximately responsible for carcinogenesis cannot be precisely determined. There are diverse chemical substances, some of the simplest structure, which, though not carcinogens in the strict sense of the term, do give rise to sarcoma in a small proportion of rats at the site of injections repeated great many times for a very long period. The cases of these "circumstantial carcinogens" are about as hopeless as the spontaneous cancers for the purpose of analysing the carcinogenic mechanism.

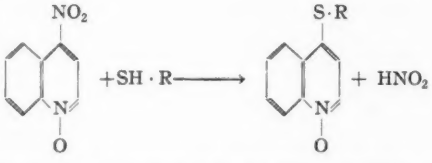
The understanding of the mechanism can be hoped for only through studies of a model case, in which carcinogenesis can be brought about fairly rapidly and with regularity, by means of a chemical agent of sufficiently simple reactivity. The recently demonstrated carcinogen, 4-nitroquinoline N-oxide, may serve as such a model as required (1, 2).

4-Nitroquinoline N-oxide, first synthesized by Ochiai and his co-workers (3), has been known to be fungicidal and mutagenic to *Aspergillus* (4) and to possess a distinct anti-cancer action (5), but it was not until 1957 that the substance was demonstrated, by the skin-painting method, to be a carcinogen of potency approaching that of the most powerful of hydrocarbons (1). This finding was of special interest, not so much because the substance belonged to a hitherto unsuspected chemical group, but more on account of its relatively simple initial reactivity.

It was soon discovered that the nitro radical at the position 4 was absolutely necessary for the carcinogenic action of 4-nitroquinoline N-oxide, all the derivatives lacking this radical being non-carcinogenic. The derivatives with C_2H_3, CH_3, or Cl at the positions 2 or 6 were found to be of about the same carcinogenic potency as the original molecule. It was also found that 4-nitroquinoline was

without the action, but the role of O-atom attached to nitrogen here may be merely to affect the reactivity of the nitro radical. Thus, studies on the relation between carcinogenicity and chemical structure easily established that the nitro radical at the position 4 may be the only part of this molecule which is primarily concerned in its carcinogenic action (2).

Discussions of structure-carcinogenic activity relationship are apt to become meaningless without adequate knowledge of the reactivity of the structure. Neither is it very enlightening merely to know that a carcinogenic molecule possesses a highly reactive center, unless we know the precise nature of the reaction which such a center undergoes. In the case of 4-nitroquinoline N-oxide, it is known that, through the strong polar effect of the N-oxide group, the polar nitro radical at the position 4 is very active and is most easily substituted by nucleophilic substances. Generally, nitro compounds are detoxified *in vivo* by the reduction of nitro radical, but Okabayashi (6) showed, through spectrographic studies, that there is no reduction of nitro radical in the reaction of 4-nitroquinoline N-oxide with thio-compounds. The reaction is that of substitution with SH group, which takes place most readily within the physiological pH range and without enzymatic intervention.



Okabayashi also reported that under similar experimental conditions, 4-nitroquinoline N-oxide did not react with COOH or NH_2 radical.

These results were confirmed and extended by Endo (7), in our laboratory, who found that those quinoline derivatives demonstrated to be carcinogenic generally showed stronger reactivity of their nitro radical with SH compounds than those that are non-carcinogenic. He also showed that no reaction whatever took place between 4-nitroquinoline N-oxide and amino acids, nucleotides, as well as nucleic acid under the experimental condition in which the former substance so immediately reacted with SH compounds.

In view of the above described facts we are reasonably certain that ***the very first chemical reaction which 4-nitroquinoline N-oxide undergoes, upon being introduced into living animal body, may be the substitution reaction with SH compounds.*** There seems to be little chance of other reactions taking place before or simultaneously. Since this substitution reaction is expected to yield (A) nitrous acid, and (B) 4-thio derivatives of quinoline N-oxide as the only possible immediate reaction products, these substances were tested for possible carcinogenicity. As

it turned out, neither of them can be shown to be carcinogenic, and at the same time 4-nitroquinolie N-oxide, when tested by subcutaneous injection, was discovered to show such a characteristic carcinogenic behavior as to strongly suggest that it may be the substitution ***reaction itself*** that plays the deciding role in the carcinogenic mechanism.

In the present paper, descriptions of our experimental data will be followed by discussion, in which a new hypothesis is proposed to explain the proximate mechanism of carcinogenesis.

Experimental Methods

The present series of carcinogenesis experiments are based on subcutaneous injections, contrary to our previous experiments in which the classical Tsutsui technique of skin painting was used. We were obliged to adopt the subcutaneous injections because of the fact that some of the substances to be tested were insoluble in benzene. Propylene glycol, physiological salt solution, or normal humna serum were used as solvents, depending on the substance to be dissolved as well as on the purpose of experiments.

The general plan of experiments was to administer certain total amounts of the test substances in divided doses, always at as nearly the same site as practicable, in the course of certain relatively short space of time, and then to keep the animals under observation with no further treatment.*

Normal adult mice of a mixed strain were used, maintaining them on the diet of whole wheat grain with dried fish and fresh carrot supplements as usual.

In all the cases autopsy was performed when animals died, and the diagnosis of every tumor was confirmed by histological examination.

Sarcoma Production by Subcutaneous Injections of 4-Nitroquinoline N-Oxide

It is first necessary to present our data on the carcinogenic effect of 4-nitroquinoline N-oxide, when administered by subcutaneous injections. In these experiments, propylene glycol was used as solvent. Propylene glycol is known to be

* In subcutaneous carcinogenesis experiments, repeated injections for a very long period of time should be condemned, because the practice sometimes yields sarcoma even when the substance injected, or its metabolites, are admittedly non-carcinogenic. Glucose, distilled water, etc., are examples of non-carcinogenic substances producing sarcoma under such drastic experimental conditions. Obviously it is not through the reaction between it (or its metabolites) and cell constituents that a non-carcinogenic substance sometimes gives rise to sarcoma. The paradox is explainable as resulting from non-specific cellular derangement, which may favor the sporadic occurrence of such energy-releasing chemical reaction within the limits of the cell's own metabolism as to disturb the replication process of the cell genetic material. See later chapters of this paper.

non-carcinogenic, and not to interfere with the reactivity of 4-nitroquinoline N-oxide.

A series of four experiments were carried out, varying the total amount of 4-nitroquinoline N-oxide to be injected, namely, from 0.5 mg to 2.0 mg per mouse.

Experiment 1. In this experiment, a total of 0.5 mg 4-nitroquinoline N-oxide per mouse was administered in 5 divided doses in the course of 40 days, at the rate of one injection in 10 days. All injections were made at the same subcutaneous sites.

Three mice developed tumors at the site of injections in 120 to 180 days after the first injection. No tumor was found in other mice, in spite of the fact that 9 of them lived over 200 days after the first injection: 221, 238, 244, 273, 280, 281, 314, 338 and 354 days.

Experiment 2. A total of 1.0 mg 4-nitroquinoline N-oxide was administered per mouse in this experiment, in 5 injections with intervals of 10 days between injections.

Tumors were produced in 2 mice at the site of injections. These tumors were found 169 and 180 days after the first injection. No other mice in this experiment showed a tumor, although 3 of them lived 273, 281 and 315 days after the first injection.

Experiment 3. The total amount af 4-nitroquinoline N-oxide per mouse in this experiment was 1.5 mg, injected in 6 divided doses in the course of 50 days, with intervals of 10 days between injections.

In this experiment tumors appeared in 3 mice in 130 to 210 days. There were 3 mice surviving over 200 days, namely: 240, 259 and 334 days. None of these mice showed a tumor.

Experiment 4. In this experiment a total of 2.0 mg 4-nitroquinoline N-oxide per mouse was given in 6 injections in the period of 50 days, with 10 day intervals between injections.

Four mice developed tumors during 130 to 190 days after the first injection. Other mice failed to show a tumor, notwithstanding that 2 of them lived as long as 232 and 262 days after the first injection.

The results of the above four expriments are graphically represented in Chart 1.

Our intention in these experiments was not to determine the minimum amount of 4-nitroquinoline N-oxide necessary for the 100 percent tumor production, but was rather to discover the general pattern which may characterize the carcinogenic phenomenon due to this peculiar chemical carcinogen. It was for this reason that we limited our tests to small total doses, delivered in a small number of injections in a relatively short space of time. Practically, it was found difficult to give more than 0.25 mg of the substance in a single injection, or to repeat the injections at shorter intervals than a week or 10 days, due to the severe local

tissue injury. We are reasonably certain, however, that by continuing injections further at proper intervals, 100 percent sarcoma production would be easily attained by 4-nitroquinoline N-oxide.

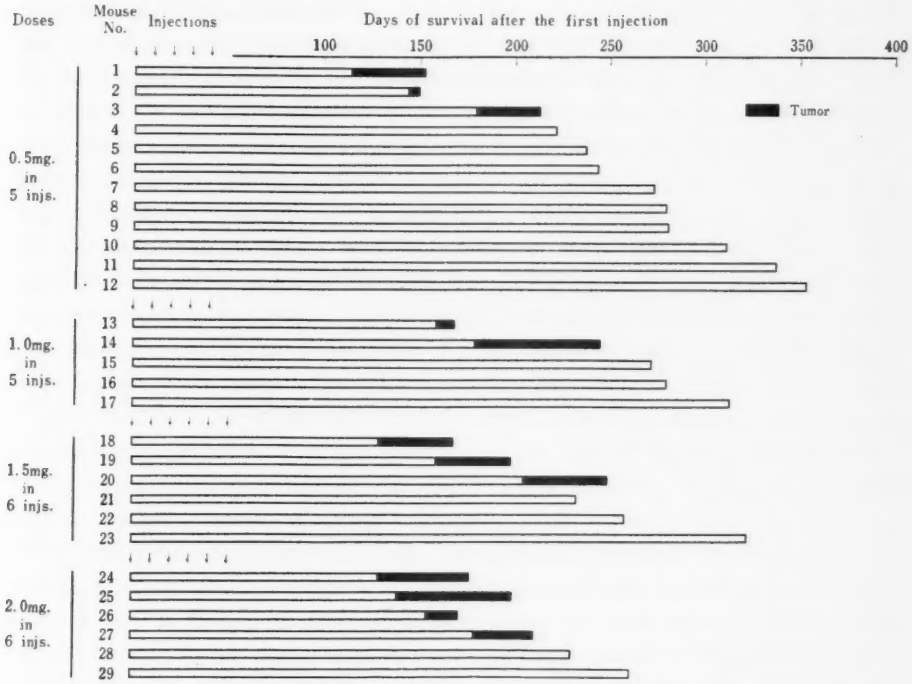


Chart 1. Sarcoma production by injections of 4-nitroquinoline N-oxide (Experiments 1～4). Only the mice developing tumors and all those that survived over 200 days are charted. The mice dying before 200 day period without tumors are excluded as experimentally ineffective, and therefore the actual tumor production rate at any given period is not represented by this chart. It is intended to show by this chart that the mice that develop tumors do so within a certain space of time (120～210 days after the first injection) and that those that fail to produce tumor by the end of this period remained free of tumor as long as they lived.

Comments on the results. The general pattern of the sarcoma production revealed by the above described experiments seems rather significant. (See Chart 1). It may be observed that sarcoma developed only in a small percent of animals but relatively early——between 120 to 210 days after the first dose of the carcinogen injection——and the animals failing to produce sarcoma during this critical period remained free from tumor for the rest of the observation period, up to 300 days or more.

This cannot be ascribed to the weakness of the carcinogen as originally adminis-

tered, since when tumors are produced, they appeared quite early. It is well known that when a powerful carcinogenic hydrocarbon is injected in decreasing doses, there is a corresponding prolongation of the latent period along with the falling off of the final tumor incidence. There is, therefore, a striking difference in the carcinogenic behavior between the hydrocarbons and 4-nitroquinoline N-oxide, and this difference can be accounted for by the relative innertness and stability of the former, contrasted to the extremely rapid reactivity of the latter.

It looks as if the doses of 4-nitroquinoline N-oxide delivered made a "hit" in a small percent of animals, while they did not take effect in others. This peculiar mode of carcinogenic action of 4-nitroquinoline N-oxide can be interpreted on the basis of the rapidity with which the substance reacts when introduced into animal body, and as indicating that, after this first reaction had taken place, the resulting metabolites are all non-carcinogenic. Presumably, tumors are produced only in cases in which the substance in its original active form has penetrated into the cells, while the bulk of the substance injected is rapidly reduced into non-carcinogenic metabolites before it had a chance to reach the target.

Pathological findings. In all the mice in the above experiments there was a marked local change at the site of the injection, the main feature being necrosis with accompanying tissue reactions. The necrosis usually became noticeable within 10 days to 2 weeks after the first injection. The area over the necrosis may even become denuded in few exceptional cases after repeated doses, though in the majority of mice the changes were not so severe There was no sloughing out of the necrotic material. After the injections were discontinued these evidences of local tissue injury gradually subsided, tending towards scar formation and contractive adhesion.

When tumors developed they were distinctly palpated as small, hard nodules at the site of injections and were generally noticeable as small protrusions as mice were inspected. The tumors generally grew rapidly. There was no macroscopically recognizable metastasis.

Tumors were examined microscopically. They included a few rhabdomyosarcomas, and all the rest were diagnosed as fibrosarcoma. Three outstanding histological types are illustrated in Plate I, Figs. 1, 2 and 3. There was a noteworthy case of very small dermal fibrosarcoma, measuring perhaps 0.5×0.3 cm or less, suggesting its origin from a minutest amount of 4-nitroquinoline N-oxide accidentally deposited intradermally in the course of subcutaneous injections.

4-Nitroquinoline N-Oxide mixed with Normal Serum

From the theory that the carcinogenic action of 4-nitroquinoline N-oxide may be determined by its first chemical reaction ***in vivo***, it may be predicted that if

the substance is given a chance to undergoe the reaction before being introduced into the animal body there will be a reduction or loss of its original carcinogenicity, depending on the completeness of that reaction.

In this experiment we examined the validity of this prediction by testing the effect of injections of 4-nitroquinoline N-oxide mixed with normal serum, assuming that normal serum contains the necessary reactants which may be involved in the *in vivo* reaction. This procedure should increase the chance of the chemical reaction in question taking place before the carcinogen penetrates into the cell, and so should decrease the yield of tumors, if our theory is correct.

Experiment 5. Four mg of 4-nitroquinoline N-oxide was mixed in 2 cc of normal human serum in the form of a homogeneous suspension. The mixture was allowecd to stand at room temperature for 30 minutes and then injected subcutaneously at the same site into mice as in the preceding experiments. Injections, 0.1 cc each, were repeated 6 times in the course of 50 days, *i. e.*, a total of 1.2 mg of 4-nitroquinoline N-oxide per mouse. Some local necrosis was evident at the site of injections, which eventually healed.

Experiment was started with 20 mice and 10 of them survived over 200 days after the first injection: 210, 213, 220, 224, 230, 230, 239, 242, 251 and 272 days. There was no tumor in any of the mice.

The result of the above experiment demonstrated that the carcinogenicity of 4-nitroquinoline N-oxide became very rapidly lost or reduced when the substance was allowed to remain *in vitro* mixed with serum for a very short time. This fact can only mean that the very first reaction which 4-nitroquinoline N-oxide underwent not only interfered with its carcinogenicity but also produced no chemical substance (reaction product) which was carcinogenic.

Our prediction has been fulfilled, and it is indicated that only the first chemical reaction which 4-nitroquinoline N-oxide undergoes is implicated in the carcinogenic effect of the substance. We strongly suspect that this first chemical reaction may be the substitution with sulphydryl compounds. It is not clear that the sulphydryl contents of normal human serum can account for the complete substitution, but even a partial substitution may be quite sufficient to reduce the amount of the active molecules penetrating into the cells so as to drastically reduce the final tumor yields.

Nitrous Acid and Sodium Nitrite

Nitrous acid is one of the two kinds of chemical products of the substitution reaction of 4-nitroquinoline N-oxide with sulphydryl compounds. It has been widely used experimentally in the studies of the inactivation of biologically active proteins, such as enzymes, hormones, toxins and viruses (8). Regarding the mode

of action of nitrous acid, it is known to deaminate aliphatic amino groups of amino acids, oxidize such groups as sulphydryl, and to diazotize phenolic hydroxyl groups. The deamination by HNO_2 of some components of nucleic acid has also been described (9). It may also be recalled that nitrite is the first of the so-called chemical mutagens (for *Aspergillus*) (10), which renders it all the more important to test the carcinogenicity of nitrous acid.

Experiment 6. Nitrous acide was produced by the reaction,

$$NaNO_2+HCl \longrightarrow NaCl+HNO_2.$$

Experimentally, 27.6 mg of sodium nitrite was dissolved in 4 cc of 1/1,000 normal hydrochloric acid, i. e., approximately equimolecular ratio, and it was immediately injected subcutaneously into mice in 0.2 cc doses. Injections were repeated 9 times about one week apart in the course of 60 days, at nearly the same site as practicable in the right inguinal region. Since $NaNO_2$ is composed of 33.32% Na and 66.67% NO_2, the total dose per mouse may be estimated to be about 7 mg as NO_2 in this experiment.

No tumor developed in any of the mice during the entire period of this experiment. Eight mice survived over 200 days after the first injection, namely: 229, 236, 244, 262, 265, 276, 313 and 341 days.

Experiment 7. An additional experiment was carried out with sodium nitrite, using 1% solution in distilled water, injecting in 0.1 cc doses subcutaneously 12 times in the course of about 90 days. The total amount of sodium nitrite injected was about 12 mg per mouse.

None of the mice showed a tumor. Nine mice survived over 200 days after the first injection: 207, 230, 241, 249, 262, 282, 292, 302 and 342 days.

In spite of the presumptive possibility, these experimental results indicated that neither nitrous acid nor sodium nitrite has any demonstrable carcinogenic action.* It is quite clear at least that the carcinogenicity of 4-nitroquinoline N-oxide cannot be accounted for by the dissociated nitrous acid.

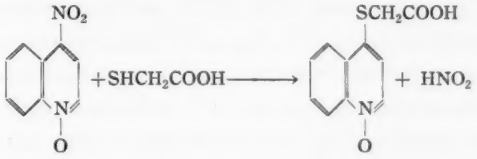
4-Thioglycolylquinoline N-Oxide

From the generalization that quinoline N-oxide molecules without nitro radical

* In pronouncing nitrous acid as non-carcinogenic, we do not lose sight of the fact that there are many instances of non-carcinogenic substances producing sarcoma in a small percent of rats when injected great many times into a subcutaneous site for a very long period of time. Subcutaneous tissue of the rat seems to be peculiarly susceptible to sarcoma production, and we feel like predicting that persistent efforts with nitrous acid will yield sarcoma, especially in rats. In such an eventuality, nitrous acid will belong to the class of what we now call "circumstantial carcinogens", which produce carcinogenic effect not by virtue of their own reaction, or of their metabolites, bnt indirectly by giving rise to chemical reactions within the cell which are conducive to producing stray energy. See footnote, p. 3.

at the position 4 are non-carcinogenic (2), it follows that 4-nitroquinoline N-oxide loses its carcinogenicity as soon as its nitro radical dissociates itself from the molecule. That the position 4 is then immediately filled through S-linkage cannot be expected to change the matter, and such products of substitution reaction are non-carcinogenic would seem to be a logical assumption. Nevertheless, we decided to subject this assumption to experimental test.

4-Thioglycolylquinoline N-oxide was selected as a model for this test, because of the relative ease with which this derivative can be prepared.



Experiment 8. 4-Thioglycolylquinoline N-oxide was made into a fairly stable emulsion with propylene glycol at the rate of 10 mg per 2 cc and was injected into mice in the usual way. The total amount of the N-oxide was 7 mg per mouse, given in 9 weekly injections in the course of 56 days.

No tumor appeared in any of the mice in this experiment, although 13 mice lived over 200 days after the first injection: 211, 233, 243, 248, 277, 285, 287, 287, 292, 307, 313 and 402 days.

The lack of carcinogenic action in 4-thioglycolylquinoline N-oxide may be regarded as proven.

Interpretation of the Results

Our investigation as described above tended to prove conclusively that it may be the substitution reaction itself, not any of the chemical products of the reaction, that is proximately responsible for the carcinogenic action of 4-nitroquinoline N-oxide. It is conceivable that, after the substitution had taken place, nitrous acid might produce some change in cell protein or nucleic acid, but it is quite clear that these processes, even if proved actually to take place, cannot account for the cancerization, since nitrous acid itself is non-carcinogenic.

It is conceivable that nitrous acid may change the nature of the bases composing the nucleic acid of the duplicant, though apparently such alterations as can be so produsedare insufficient for cancerization, if not unrelated to it. Since purely chemical action can produce only some specific types of alterations, one must assume the participation of a large number of active chemical substances in order to produce a multitude of alterations. This concept is difficult to support, at least from the case of 4-nitroquinoline N-oxide.

An important consequence of the substitution reaction of 4-nitroquinoline N-

oxide would be the possible fixation of quinoline N-oxide through sulphur linkage to protein. In view of the fact that SH group forms the prosthetic group of many enzymes, this fixation may at first sight appear to be significant, especially when considered in relation to the enzyme deletion theory of carcinogenesis. Closer examination, however, shows how unlikely it is that such a fixation has anything to do with the carcinogenic process.

The consumption of SH group may be expected to result in the suppression of SH-enzymes, which in turn may produce inhibition of glycolysis. Fukuoka, Sugimura and Sakai (11) indeed demonstrated that 4-nitroquinoline N-oxide markedly inhibits glycolytic action of cancer cells, which explains the anti-cancer action of this substance. How could this mechanism lead to the production of cells (malignant) with high glycolytic activity? Moreover, it is difficult to imagine how a binding with SH-containing protein could produce an irreversible change in cell genetic material (nucleic acid), to which SH group bears no direct relation.

These considerations leave to us nothing to implicate in the carcinogenic mechanism but the energy released by the substitution reaction.

It has generally been supposed that the earcinogenic effect of chemical carcinogens may be due to their conversion into active metabolites, and this idea seems to have long guided many of the studies by previous workers. The immense amount of the work on the metabolism of carcinogenic substances so far piled up, however, failed to disclose any active metabolite. This situation is fully realized by Haddow (12) when he generalized that "Much of the work.....suggests that it is not the hydrocarbon or matabolite which is the proximate carcinogenic agent, but rather the energy released during the transformation from one metabolite to another."

In our studies with 4-nitroquinoline N-oxide, we seem to be forced to deduce that the energy released by its reaction may be the proximate cause of carcinogenesis, because of the lack of any acceptable alternative. In this deduction we assumed that the substitution reaction with SH compound may be the only reaction which needs to be considered, the assumption that there may be no other reaction which takes place either before or simultaneously. However, the very rapid loss of the carcinogenic activity of 4-nitroquinoline N-oxide when it is mixed with normal serum clearly indicates the deciding role of the very first chemical reaction, and whether or not that reaction involves only the substitution with sulphydryl compound may perhaps be of no essential importance in the bioenergetic interpretation of the carcinogenic mechanism.

A Speculative Hypothesis

Regardless of whether we call it somatic mutation or not, the indisputable fact is that in carcinogenesis a new cell race is produced, which, though retaining many characteristics of the original normal cells, has lost responsiveness to normal

growth regulating mechanism (autonomous growth), and acquired great capacity for glycolysis and for synthesizing toxohormone, and these peculiarities are transmitted to the cell progeny. It is therefore absolutely necessary to assume that genetic material or hereditary determinat of somatic cell is modified in some manner. For the sake of convenience we may call this genetically critical, self-duplicating fiber or template of somatic cell the "duplicant", after Nothdurft, Butenandt, Druckrey, von Euler, etc. (13, 14, 15, 16).

Difference of opinions arises when we come to consider the mechanism by which the modification of the duplicant may be brought about. Many workers seem to have taken it for granted that such alteration occurs following a chemical combination with carcinogen, that is, carcinogen first becomes fixed to the critical cell component before it exerts its action. Such a fixation, however, seems on longer essential, now that it has been shown that with water and its structure as the matrix, many kinds of interaction can take place between molecules without their even touching one another (Szent-Gyorgyi, 17).

It may be recalled that Schrödinger (18) suggested that irreversible and heritable changes (mutation) which occur in the cell might be due to quantum jumps in the gene molecule. He showed how this sort of "isomeric" transitions can take place between two configurations, which are not neighboring, over intervening configurations which have a greater energy than either, and which normally act as barriers. Iversen and Arley (19) interpreted the then available experimental findings from the point of view of the quantum theoretical "hit" theory, the basic assunption of which is that the molecule, which constitutes "cancer control center" (duplicant in our terminology) is excited to making a quantum transition to a new state by reaction between it and a molecule of the applied agent. This new state is thought of as a different spacial arrangement of the various atoms comprising the molecule i. e., an "isomeric" form.

Following a similar line of thought, it is possible to suggest that the energy released is absorbed by the electrons of the duplicant molecule, which become excited to making quantum transitions, resulting in some intramolecular rearrangement of the configurations, and some of the rearranged forms thus produced are the cancerized duplicants.

One drawback here is that we have no idea as to the amount of energy that is required to produce the postulated quantum jumps in the duplicant molecule. There is, in addition, a positive difficulty in explaining the well known different susceptibility of different cells to one and the same carcinogen. This difficulty can be set aside by introducing as a deciding factor the different frequencies of duplicant replication taking place in different cells. A cell in the proliferative state must contain more duplicants in the process of replication than a resting

cell, and so should offer a greater chance for the released energy to take effect. This may also help to explain why cancerization does not occur more frequently, considering the conceivable common occurrence of abnormal energy-releasing chemical reactions in cellular system. This idea can be further developed by assuming, with Druckrey (15), that a multitude of duplicants must be modified in order to effect the cancerization of a cell., since the chance of stray energy inducing this final effect may be decreased by the power of the number of the duplicants that must be modified.

We, therefore, propose the following hypothetical mechanism as best satisfying all the requirements:

The energy released disturbs the normal replication process of the duplicants, by misdirecting the sequence of configurations which are being formed alongside the template, or by preventing one or more of components from joining up to the forming chain.

The energy-releasing chemical reaction must occur in sufficient proximity to the site of duplicant replication to insure the absorption of at least a part of the energy by the electrons of the configurations which are in the process of replication.

The necessary amount of the energy required at the moment of action may be predicted to be not more than the bond energy among the components concerned, if it is supposed merely to misdirect, but more than the bond energy if it is to prevent, the combination of the components.

Since this mechanism operates upon the formative stage of the duplicant in the course of its replication, it may be expected to produce the result much more easily and with much less energy than the mechanism, which is supposed to attack the already formed duplicant.

The postulated configurational transitions are not to be supposed to take place always at the same loci of the duplicant molecule. It is therefore necessary to assume that a multitude of duplicants must be affected variously and that cancerization results from the summation of all these irreversible changes, as suggested by Druckrey (15), von Euler (16), etc.

It must be clearly understood that the suggested mechanism is purely speculative. We do not know the amount of energy which is released by the reaction of 4-nitroquinoline N-oxide with SH compound, for instance. Neither do we know exactly how much energy is required to produce the postulated misconstruction of duplicant. Our hypothesis may fail if, upon actual calculations, the energy released is discovered to be less than that required to disturb the replication process.

General Discussion

It must be confessed that in the present study our minds have been dominated by the idea that fundamentally there is but one universal primary mechanism for carcinogenesis, the idea that no matter how many superficially different agents there may be to produce cancerization of the cell, all of them will ultimately converge upon a single proximate mechanism, which may be called the "real" or "fundamental" cause of cancer, in contrast to the multitudinous "apparent" or "superficial" causes. Erroneous impression exists among investigators that elucidation of the carcinogenic mechanism of any one of the various chemically unrelated carcinogens can have at best only a very limited applicability. We believe, however, that if the mechanism worked out for one chemical group of carcinogens is found to be incapable of general application, it should be taken to indicate that the solution of the problem does not lie in that direction. Other mechanisms must be sought before suggesting different mechanisms for different carcinogens.

At the risk of repetition, it may be stated that the only mechanism that can be reasonably postulated on the ground of the present study with 4-nitroquinoline N-oxide seems to be related to the energy released by its reaction, whereby mutational changes in duplicants can be assumed to take place through disturbance of the replication process. Once such a somatic cell change has taken place, the rest of the processes leading to the formation of clinically recognizable cancer may follow, though subject to considerable modification by various factors involved in the cell environmental conditions, for example, presence of absence of promoting influence (Rous, 20).

In formulating the hypothesis of the misconstruction of duplicants by the energy released through chemical reaction, we have been tacitly under the influence of Muller (21) and others, who visualize the mechanism of gene mutation as resulting from an accidental encounter between the gene molecule and some energy-conveying particle. Needless to add that we refer here only to the energy released intracellularly by some abnormal (abnormal to the cell) substance, or through the blockage at some point of the normal cycle of energy transfers. One of the strengths of this hypothesis consists in the fact that any kind of molecules can be carcinogenic if it releases right amount of stray energy at right intracellular sites. All the known chemical carcinogens can be expected to do this at some stage of their transformations from metabolite to matabolite. Another point in favor of this hypothesis is that a misconstruction of the duplicant molecule can very well occur accidentally in the course of its self-duplication by the action of some stray energy. The spontaneous cancerization, which occurs ***in vivo*** as well as ***in vitro***, without the aid of any detectable external influence, can be explained under this hypothesis.

The proximate carcinogenic mechanism postulated in the present hypothesis

constitute the primary ***sine qua non*** for the production of cancer, and further understanding of physical processes involved may have to be preceded by some epochal progress in the application of bioenergetics to cell research. However, it is to be noted that Watson and Crick's (22) proposed model of desoxyribonucleic acid has already furnished a plausible basis for specificity, replication and mutation of the gene, and since the duplicant must be a nucleoprotein derivative of ribo- and / or desoxyribonucleic acid, there is every reason to believe that the definite knowledge of the duplicant, and eventually of the difference between normal and cancerous duplicants, may be within our reach.

Finally, it may be suggested, in agreement with the view expressed by von Euler (16), that cancer virus may be the cancerized duplicant itself. From this point of view the cell-free transmissibility of tumors may depend on the ability of cancerized duplicant, separated from one tumor, to penetrate into the cell of recipient animal and to proliferate there. The Rous virus may be an example of such competent duplicants, while in the majority of mammalian tumors, their duplicants are unable either to survive the separation from tumor cell, or to penetrate into and proliferate in the cell of the inoculated animal. By associating it with virus, we may receive additional impetus for examining the problem of the duplicant in the term of a nucleoprotein derivative.

Summary

The mechanism of carcinogenic action of 4-nitroquinoline N-oxide was analyzed based on the assumption that the substitution reaction with SH compound may be the first reaction which this substance undergoes ***in vivo***. Experimental results indicated that this reaction itself, but not any of the chemical products of the reaction, may be the proximate cause of carcinogenesis, leading to the hypothesis that the energy released by the reaction produces misconstruction of the genetic determinant of the somatic cell (duplicant) by disturbing its normal replication process.

This hypothesis was discussed in relation to the universal proximate mechanism applicable to all the forms of carcinogenesis.

We wish to express our thanks to Professor E. Ochiai, Pharmaceutical Institute, University of Tokyo, and Dr. S. Sakai, Laboratories of the Kaken Chemicals, Ltd., for the donation of 4-nitroquinoline N-oxide. The sample of 4-thioglycolylquinoline N-oxide was prepared for us by Dr. H. Endo of our Institute, to whom our thanks are due. We are also indebted to Dr. K. Oota of our Institute for his kindness in examining most of our histological specimens.

This investigation was supported in part by a grand-in-aid from the Japanese Ministry of Education.

References

1. Nakahara, W., Fukuoka, F., and Sugimura, T. Gann, **48**: 129 (1957).
2. Nakahara, W., Fukuoka, F., and Sakai, S. Gann, **49**: 33 (1958).
3. Ochiai, E., and Sei, S. Jour. Pharmaceut. Soc. Japan, **65**: 18 (1945).
   Ochiai, E., and Okamoto, T. Jour. Pharmaceut. Soc. Japan, **70**:384 (1950).
4. Okabayashi, T. Jour. Ferment. Technol., **33**: 513 (1955).
5. Sakai, S., Minoda, K., Saito, G., Akagi, S., Ueno, A., and Fukuoka, F. Gann, **46**: 605 (1955).
6. Okabayashi, T. Jour. Pharmaceut. Soc. Japan, **73**: 946 (1953).
7. Endo, H. Gann, **49**: 151 (1958).
8. Harriott, R. M. Advances in Protein Chem., **3**: 169 (1947).
9. Bendich, A. The Nucleic Acids (Chargaff and Davidson), **1**: 81 (1955).
10. Thom, C. Proc. Nat. Acad. Sci., **25**: 329 (1939).
11. Fukuoka, F., Sugimura, T., and Sakai, S. Gann, **48**: 65 (1957).
12. Haddow, A. Physiopathol. of Cancer (Homburger and Fishman), 441 (1953).
13. Nothdurft, H. Zeitschr. f. Krebsforsch., **56**: 234 (1948).
14. Butenandt, A. Verh. deutsch. Ges. inn. Med., **55**: 342 (1949).
15. Druckrey, H. Strahlentherap., **83**: 597 (1950).
16. von Euler, H. Gann, **49**: 69 (1958).
17. Szent-Gyorgi, A. Bioenergetics. Academic Press (1957).
18. Schrödinger, E. What is Life? The Physical Aspect of the Living Cell. Cambridge Univ. Press (1944).
19. Iversen, S., and Arley, N. Acta pathol. microbiol. Scandinav., **27**: 773 (1950).
20. Rous, P., and Kidd, J. G., Jour. Exper. Med., **73**: 365 (1941).
    Friedenwald, W. F., and Rouse, P. Jour. Exper., Med., **80**: 101 (1944).
21. Muller, H. J. Symposium on Radiobiol., Nat. Acad. Sci., 296 (1952).
22. Watson, J. D., and Crick, F. H. C. Nature, **171**: 964 (1953).

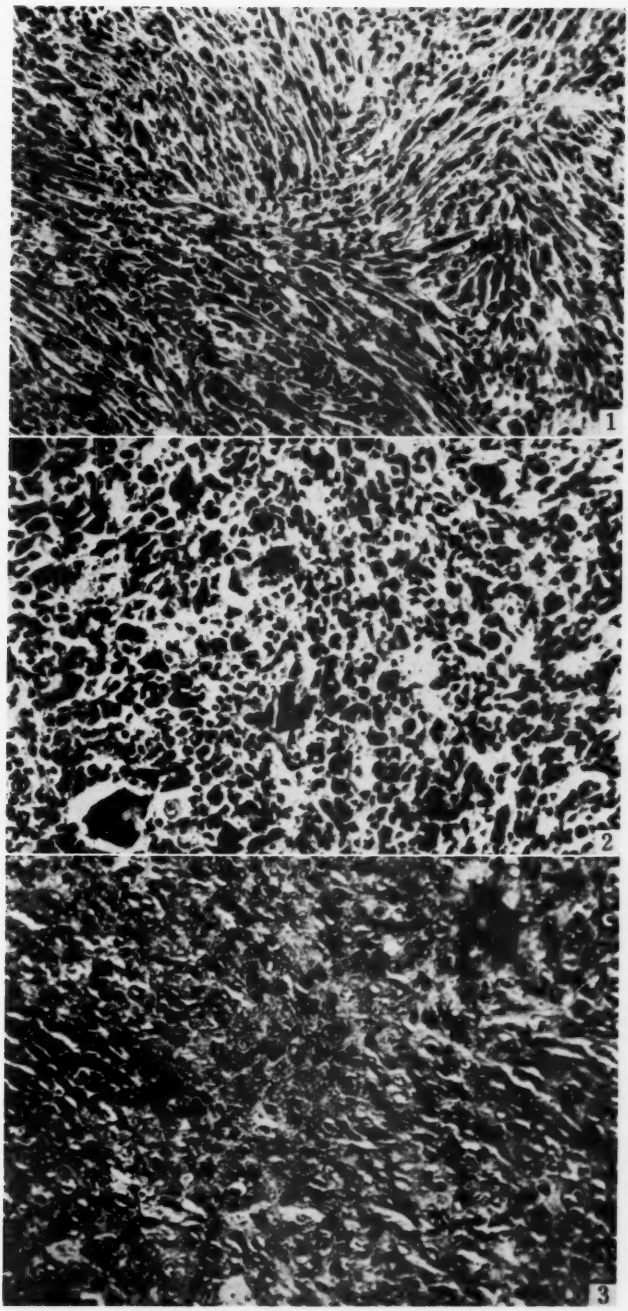
Explanation of Figures (PLATE I)

Photomicrographs showing three examples of fibrosarcomas produced by subcutaneous injections of 4-nitroquinoline N-oxide. (The total amount of carcinogen injected and number of days after the first injection in parenthesis).

Fig. 1. Fibrosarcoma of spindle cell type (0.5 mg: 150 days).

Fig. 2. Fibrosarcoma of polymorphous cell type, with numerous giant cells (1 mg: 169 days).

Fig. 3. Fibrosarcoma of very short cell type, simulating carcinoma. This is a very rare type, represented by a single case in the present series (1.5 mg: 250 days).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[GANN, 50, 17~21; March, 1959]

ON THE MECHANISM OF RADIATION CARCINOGENESIS

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INTRODUCTION

In a separate paper (1), we reported experiments which indicated that the carcinogenic action of 4-nitroquinoline N-oxide may be due to the nucleophilic substitution reaction *itself* which this substance so readily undergoes, but not to any of the chemical products of the reaction. This inevitably led us to postulate that the energy released by the reaction may produce mutational change in cell duplicants by disturbing the normal processes of their replication, after the manner generally assumed for the gene mutation (Muller, 2). We also showed how this speculative hypothesis can be applicable to all the known forms of carcinogenesis. In the present paper, we wish to place on record other experimental results which seem to show that an analogous situation may hold in the case of radiation carcinogenesis.

It is generally accepted that radiobiological effects are produced through the transfer of radiation energy to water, which results in the dissociation of the water molecule into free radicals and in the formation of hydrogen peroxide, which can react with the terget molecules It has been suggested, though without any direct evidence, that carcinogenesis may involve a mutation-like change which may be brought about by free radicals (3).

We have found, however, that neither hydrogen peroxide nor free radicals derived from it can be shown to be carcinogenic, when tested on mice by means of repeated subcutaneous injections. If these negative results can be accepted on their face value, it would seem, as in the case of 4-nitroquinoline N-oxide, that it is not the chemical products (hydrogen peroxide and free radicals) that are responsible for the carcinogenic effect of radiation, which, then, can be ascribed either to the direct action of radiation energy or to the energy released by the dissociation of the water molecule, or both, on the replication processes of the duplicant molecule.

MATERIAL AND METHODS

Hydrogen peroxide was used at the concentration of 0.5%, diluting a commercially available 3% solution with physiological salt solution. This concentration was chosen as probably most suitable after repeated trials of various lower and

higher concentrations. It was injected in 0.1 cc doses. In one experiment, with the hope of possibly facilitating tumor production, purified diatomaceous earth (kieselguhr) was sometimes added to hydrogen peroxide solution at the rate of about 0.1 mg per 0.1 cc.

In some experiments, for the purpose of protecting against the action of catalase, a trace of 1% solution of hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) was added to the diluted hydrogen peroxide solution.

It is well established that free radicals are formed by the action of ferrous ions on hydrogen peroxide, as in the following scheme (Weiss (4)):

$$H_2O_2 + Fe^{++} \longrightarrow OH^{\cdot} + Fe^{+++} + OH^{-}$$
$$H_2O_2 + OH^{\cdot} \longrightarrow H_2O + HO_2^{\cdot}$$
$$H_2O_2 + OH_2^{\cdot} \longrightarrow O_2 + H_2O + OH^{\cdot}$$

For the purpose of our experiments, solution containing free radicals was prepared by taking up in an injection syringe and mixing therein a solution of 0.2 mg of $FeSO_4 \cdot 7H_2O$ in a smallest amount of water, and 0.5% H_2O_2 solution, at the ratio of about 0.01 mg $FeSO_4 \cdot 7H_2O$ to 0.3 cc H_2O_2 solution. The mixture was injected promptly, in 0.1 cc amounts, allowing in no case more than 30 seconds after mixing.

Normal adult mice of a mixed strain were used in the experiments. They were maintained on our usual diet of whole wheat grain, supplemented with dried fish and fresh carrots, throughout the experimental period.

Injections were made subcutaneously, always at nearly the same site as practicable in the right inguinal region.

Experimental Results

Experiments carried out fall into four major groups, according to the substances added to hydrogen peroxide solution. In the following lines experimental results will be given of each group in the terms of the number of injections and the space of time in which they were given, and the number of mice surviving long enough to be experimentally effective, with records of the actual number of days of survival for each mouse.

Mice dying before 200 days are not noted here; needless to say that they were all free of tumor at autopsy. The main difficulty in these experiments was in keeping the mice alive for a long enough period for the evaluation of the possible carcinogenic effect of the treatments, which in some cases were rather drastic.

Hydrogen peroxide alone:

Injections were repeated three times a week for as long as the mice lived. Five mice survived long enough to be experimentally effective: 201, 204, 248, 291 and 302 days. No tumor was found in any of them.

Hydrogen peroxide plus ***Kieselguhr***:

Experiment was conducted in the same way as above, except that kieselguhr was added to the hydrogen peroxide solution on four occassions during the first 3 months. There were 6 mice experimentally effective, surviving 238, 267, 267, 275, 280 and 309 days. None of the mice showed any tumor.

Hydrogen peroxide plus *Hydroxylamine*:

In these experiments, 1% solution of hydroxylamine in distilled water was added to hydrogen peroxide solution at the rate of 1/20 the volume of the latter. Due perhaps to the toxicity of hydroxylamine it was difficult to continue the injections for a long time. In one series 11 injections were given in the course of first 30 days and then the injections were discontinued. Two mice survived 220 and 240 days. In the other series, 20 injections were given during the first 60 days, and 2 mice survived 254 and 292 days. Tumor did not appear in any of these 4 mice.

Hydrogen Peroxide plus *Ferrous Sulphate*:

These experiments were carried out in a series of seven, experimental conditions being slightly modified in each case as follows:

(1) Six injections were made in the first 12 days, and the mice were left untreated thereafter. Four mice survived 268, 316, 316 and 331 days.

(2) Ten injections were given during the first 20 days, 5 mice surviving 228, 247, 255, 260 and 270 days.

(3) Ten injections in the first 30 days, 4 mice surviving 270, 282, 328 and 330 days.

(4) Twelve injections in the first 23 days. In this group 10 mice survived 265, 280, 280, 280, 281, 301, 311, 320, 325 and 332 days.

(5) Forteen injections in the first 30 days. Four mice survived 280, 291, 305 and 325 days.

(6) Seventeen injections in the first 70 days, only one mouse surviving 227 days.

(7) In this last experiment injections were continued 3 times a week for a period of 150 days, and mice were kept under observation without further treatment. Two mice only survived long enough to be experimentally effective: 200 221 days.

None of the 32 mice in the above series showed tumor.

Comments: In all these experiments the early tissue changes at the site of injections consisted of local necrosis of varying extent and granulative reaction, often with leucocytic infiltration suggestive of local infection. Small abscesses were sometimes encountered. In some cases, especially around 50 to 100 day period, the tissue reaction seemed vigorous, with signs of regenerative cell proliferation, but such changes apparently did not go over to neoplasia. The changes later seemed to tend to scar formation. When ferrous salt was included in the inoculum the site of injections was stained brownish yellow. The general systemic

changes were essentially atrophic, liver and spleen being generally reduced in size.

We were impressed by the fact that during the course of the experiments the local tissue at the site of injections was maintained in the state of chronic irritation, with necrosis, infiltration, regeneration, and sometimes suppuration, and yet not a single tumor developed among some 30 mice which survived 200 to over 300 days. Apparently, the experimental conditions lacked the essential factor without which carcinogenesis does not occur even with long continued local irritation.

Discussion

Although there is as yet indadequate evidence to show that free radical reactions occur in normal metabolic processes, and biochemists generally are more or less skeptical of the concept that free radical chain reaction of the type that is known in simple organic chemical system occur in biochemical processes, there can be no doubt that in the effect of radiation free radical reactions do occur in biological systems.

The primary products of the ionization of water are a positive water ion and an electron, and from the formation of an ion pair, two radicals result as first suggested by Weiss (5):

$$H_2O^+ + H_2O \longrightarrow H_3O^+ + OH^\cdot$$
$$e^- + H_2O \longrightarrow OH^- + H^\cdot$$

These radicals may combine to form hydrogen peroxide, molecular hydrogen and water. In biological systems in which molecular oxygen is always present, perhydroxyl radical is also formed (whereby the yield of hydrogen peroxide is increased):

$$H^\cdot + O_2 \longrightarrow HO_2^\cdot$$

The role of the free radicals in the production by radiation of mutation, and therefore also of carcinogenesis, has been strongly advocated by some authorities (3).

As to hydrogen peroxide, there have been some speculations as to its possible implication in carcinogenic processes, based on the presumed reduced activity of catalase accompanying the process. Theoretically, all biological oxidation processes——lactic acid, succinic acid, ascorbic acid, glutathione——give H_2O_2 as the end product, and the reason that it has not been possible actually to detect it has always been attributed to the presence of catalase. Unbalance in H_2O_2-catalase equilibrium in favor of the former may possibly make the effect of H_2O_2 felt. Thus, one of us (Nakahara, 1947) stated "If H_2O_2 generated in the animal body should fail to be completely decomposed, due to the reduced catalase activity, and even a small amount of it remains, it is conceivable that its action might induce malignant transformation of the cells acted upon, or produce a carcinogenic substance by acting on some cell constituent." A somewhat similar idea was

expressed by Rondoni (1952) that the reduction in catalase activity might have some bearing on the protein rearrangement in carcinogenesis, producing an accumulation of H_2O_2 and consequently an aggregating effect on certain of the protein fractions.

Notwithstanding these speculative suggestions, however, it has been impossible to demonstrate experimentally that either hydrogen peroxide or free radicals have carcinogenic action, as reported in the present paper.

It might possibly be objected that our experimental method (subcutaneous injections) might not have permited the penetration of H_2O_2 or free radicals into the cells, because of the expected great rapidity of the reaction of these agents. We can only reply by stating that the precaution we took (addition of hydroxylamine) against the attack by catalase did not change the matter. However, we consider that the constant production of local tissue necrosis in the experimental animals may be taken to indicate that the agents injected did reach the cells in biologically active form.

Conclusions

Repeated subcutaneous injections in mice of hydrogen peroxide or free radicals generated from it by ferrous salt absolutely failed to produce carcinogenic effect. These negative results are interpreted as showing that the well known carcinogenic effect of ionizing radiation may be due, not to the chemical products, but rather to the energy released by the dissociation of water molecules, and / or to the direct action of radiation energy itself.

These facts and deductions are consistent with our hypothesis that the proximate mechanism of experimental carcinogenesis may consist of the extra energy released intracellularly, whereby misconstruction (mutational changes) can be assumed to take place in the duplicant molecule through disturbance of its normal replication process.

This investigation was supported in part by a grant-in-aid from the Japanese Ministry of Education.

References

1) Nakahara, W., and Fukuoka, F. Gann, **50**: 1 (1959)

2) Muller, H. J. Symposium on Radiobiology, Nat. Acad. Sci., 296 (1952).

3) See, for general discussion, Jensen, E. V. Biological Antioxidants, 159. Josiah Macy, Jr., Foundation, New York. (1950).

4) Weiss, J. Nature, **163**: 691 (1949).

5) Weiss, J. Nature, **153**: 748 (1944)

6) Nakahara, W. Report 19th Research Group, 9th division, National Research Council of Japan, 14 (1952).

7) Rondoni, P. Boll. Oncologia, **26**: 245 (1952). Advances in Cancer Research, **3**: 171 (1955).

[GANN, **50**, 23~35, March, 1959]

EFFECT OF 4-NITROQUINOLINE N-OXIDE ON THE METABOLISM OF CANCER CELLS IN VITRO

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INTRODUCTION

Recent studies originating from our laboratories brought into prominence 4-nitroquinoline N-oxide, and certain derivatives of it, as possessing anti-cancer action (1) as well as powerful carcinogenic potency (2). The carcinogenic mechanism of this interesting class of molecules has been investigated with a definit conclusion (3). As to the mode of anti-cancer action we already reported that such of the derivatives as are anti-cancerous strongly inhibit the tumor cell glycolysis ***in vitro***, while other derivatives that are inactive against cancer fail to produce the inhibition (4). It was also demonstrated that the incorporation of ^{32}P into nucleic acids of cancer cells was markedly inhibited by anti-cancer derivatives (5), though this inhibiting action was found to be related to the presence of nitro-radical at the position 4, rather than strictly to the anti-cancer potency of the molecule.

In view of the well known high glycolytic activity as the energy yielding process of cancer cells, we ventured to presume that the glycolytic inhibition by 4-nitroquinoline N-oxide may bring about the suppression of nucleic acid and protein synthesis through interrupting this energy yielding process. In this paper our investigations concerning this problem are presented, with special emphasis on the point in the glycolytic chain of events which may be interrupted by 4-nitroquinoline N-oxide.

EXPERIMENTAL METHODS AND MATERIALS

Two quinoline derivatives were selected for this study, namely, 4-nitroquinoline N-oxide and quinoline N-oxide, representing respctively the active and inactive molecules as to the anti-cancer as well as carcinogenic activities. In most of the experiments the effects of these two derivatives were compared by adding them to various reaction systems at the final concentration of 3.0×10^{-4} mol. in Krebs-Ringer bicarbonate solution, with the aid of a very small amount of propylene glycol, the amount previously tested and found to be without any effect.

Cancer cells used were of the Ehrlich ascites carcinoma of the mouse. Tumor ascites was aspirated 7 days after intraperitoneal transplantation. Cells were washed with ice-cold 0.9 percent NaCl and contaminating red cells separated by slow centrifugation. The tumor cell pellet obtained was washed with and resuspended in the buffer solution for incubation.

Incorporation experiments were carried out as usual by incubating at 37°C the mixture of cancer cell suspension and ^{32}P or one of the labeled amino acid samples.

Glycine-2-^{14}C was obtained from the Daiichi Pure Chemicals Co., Ltd., and DL-methionine-^{35}S from the Radiochemical Center, Amersham, England. ^{14}C-labeled amino acid mixture was kindly furnished by the Institute of Applied Microbiology, University of Tokyo, where it was prepared from the protein of *Chlorella ellipsoidea* cultivated in a medium with $^{14}CO_2$ as the sole carbon source (6).

Methods for the fractionation of nucleic acids, protein, etc., after the incubation, will be described under the respective experiment. The paper chromatography of acid soluble phosphorous compounds in the cell was carried out according to the method described by Baudurski and Axelrod (13). The spots of the standard phosphorylated compounds were detected by a ferric salicylsulfonate spray described by Wade and Morgan (15).

Glycolysis was measured according to the classical Warburg method, placing in each vessel a total of 3 m*l* of solution containing glucose in the final concentration of 200 mg/d*l* and $1.0 \sim 1.6 \times 10^7$ cells, using 95 percent nitrogen and 5 percent carbon dioxide as the gas phase. In each case a 10 minutes equilibrium time was allowed, and then the evolved carbon dioxide caused by lactic acid formation in bicarbonate buffer was measured at 10 minutes intervals.

Results

Incorporation of ^{32}P into RNA and DNA

The incorporation of ^{32}P into RNA and DNA was tested under the anaerobic condition in this experiment, incubating at 37°C for one hour a reaction mixture containing 0.5 m*l* of 10 percent suspension of washed cancer cells in 4 m*l* of Krebs-Ringer bicarbonate solution, which contained glucose at the rate of 2 mg/m*l* and ^{32}P at 1 μc/m*l*. The gas phase was made up of 95 percent N_2 and 5 percent CO_2. 4-Nitroquinoline N-oxide and quinoline N-oxide were used at the final concentr-

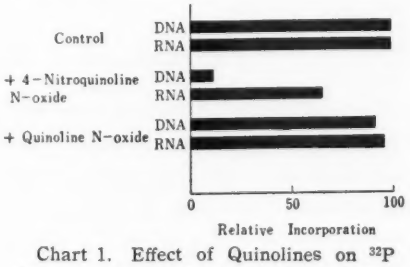


Chart 1. Effect of Quinolines on ^{32}P Incorporation into Nucleic Acids of Ehrlich Carcinoma Cells.

ation of 3.05×10^{-4}M.

After the incubation the cells precipitated with cold 1N perchloric acid were washed three times with 0.5 N perchloric acid, and extracted with ethanol-ether (3 : 1) to remove lipids. The residue was then fractionated by the method of Schmidt and Thanhauser (7), decomposing it in 1N KOH at 37°C for 20 hours, acidifing the chilled solution with 6N HCl, treating with 1/10 volume of 9N perchloric acid and centrifuging. The supernatant was designated as RNA fraction. The precipitate was washed once with 5 m*l* of 0.5 N perchloric acid and treated with 5 m*l* of 0.5 N perchloric acid at 95°C for 15 minutes, and centrifuged. This supernatant was designated as DNA fraction.

As shown in Chart 1, 4-nitroquinoline N-oxide markedly inhibited ^{32}P incorporation into RNA and DNA, but quinoline N-oxide failed to show any effect.

Incorporation into Protein of Glycine-2-^{14}C and DL-Methionine-^{35}S

The first test was made under anaerobic condition, the reaction mixture and other experimental conditions being the same as in the preceding experiment, except that glycine-2-^{14}C, 150 γ, (6,500 cpm/m*l*) was used.

After the incubation, protein fraction was prepared from the cells either according to Schneider's (8) or Schmidt and Thanhauser's method (7). In the latter case, the residue after the removal of DNA fraction was washed once with 5 m*l* of 0.5N perchloric acid and was designated as protein fraction.

Here again the results showed that the incorporation of glycine-2-^{14}C into protein

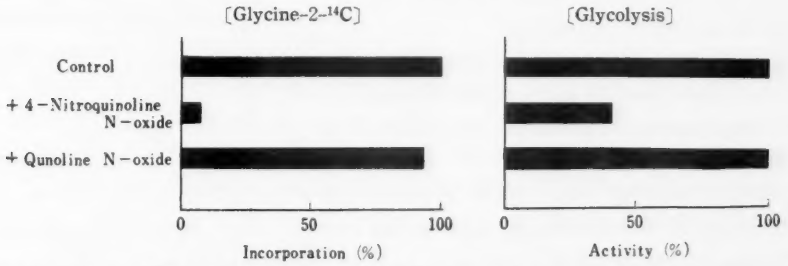


Chart 2. Effect of Quinolines on Glycine-2-^{14}C Incorporation into Protein and Anaerobic Glycolysis of Ehrlich Carcinoma Cells.

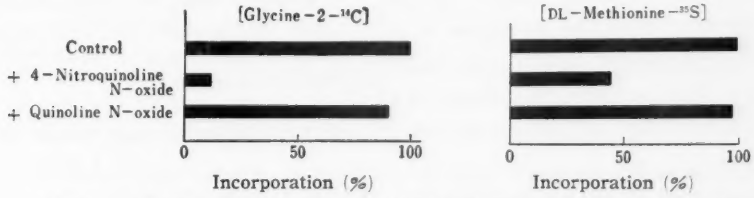


Chart 3. Effect of Quinolines on Labeled Amino Acid Incorporation into Protein of Ehrlich Carcinoma Cells. [Aerobic Condition]

was markedly inhibited by 4-nitroquinoline N-oxide, but not at all by quinoline N-oxide. The glycolysis tests carried out at the same time paralleled these results. See Chart 2.

Experiments conducted under aerobic condition also revealed similar effects. In this case the reaction mixture similar to before contained 300 γ/ml (113,000 cpm/ml) of glycine-2-^{14}C, or 708 γ/ml (25,000 cpm/ml) of DL-methionine-^{35}S. For unknown reason, the inhibiting effect of 4-nitroquinoline N-oxide was not as striking on DL-methionine-^{35}S incorporation as on glycine-2-^{14}C incorporation. (Chart 3).

Time Relation between Glycolytic Inhibition and Inhibition of Incorporation

It now becomes necessary to correlate the inhibition of glycolysis, of ^{32}P incorporation into nucleic acids, and of glycine-2-^{14}C incorporation into protein, as regards their time relations. In these experiments care was taken to allow the cancer cells in the accessory chamber to go into the mixture of isotope and quinoline in the main chamber of a Warburg vessel after a 10 minutes' temperature equilibrium time. The general experimental conditions were the same as in the first experiment.

As may be seen from Chart 4, the inhibition of glycine-2-^{14}C into protein is already well manifest before the glycolytic inhibition becomes evident. Similarly, the inhibition of ^{32}P incorporation into RNA and DNA also tended to start before the onset of glycolytic inhibition.

It is of interest to note that the inhibition of glycolysis did not appear until 40 minutes after the beginning of the reaction. 4-Nitroquinoline N-oxide is well known to rapidly undergo substitution reaction with sulphydryl compounds, generating nitrous acid, but we confirmed by another experiment that the apparent delay in the appearance of the inhibition observed here was not due to the superposition of the volume of nitrous acid to that of carbon dioxide. The volume of nitrous acid produced from the very minute amount of 4-nitroquinoline N-oxide used is actually negligible.

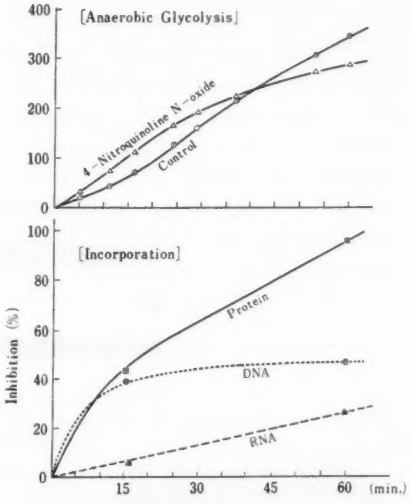


Chart 4. Effect of 2-Nitroquinoline N-oxide on Glycolysis and Isotope Incorporation into Protein, DNA and RNA of Ehrlich carcinoma Cell.

Incorporation of ^{14}C Amino Acids into Protein in Cell-free System

At this point an important departure was introduced in our study by the use

of the cell-free system, in which the energy source consisted of energy-yielding phosphates, instead of glycolysis, and the cellular elements microsome and pH 5 enzyme fractions, instead of intact cells. Effect of 4-nitroquinoline N-oxide on the incorporation of ^{14}C labeled amino acids into protein was investigated in this system.

The microsome fraction and pH 5 enzyme were prepared from mechanically disintegrated Ehrlich carcinoma cells following exactly the method described by Littlefield and Keller (9).

In this experiment, the complete system was made up of the microsome and pH 5 enzyme fractions, 2.7 mg as protein determined by the method of Lowry *et al.* (9), with phosphoenolpyruvate (PEP), 10 μM, adenosine triphosphate (ATP), 1 μM, and glucose-1-phosphate (G-1-P), 0.25 μM., All labeled amino acids obtained by hydrolysis of ***Chlorella*** protein, 75,000 cpm., was added. 4-Nitroquinoline N-oxide was used at 0.075 mg/m*l*. The total final volume of the reaction mixture was 2 m*l*, and it was incubated at 37°C in air for 60 minutes.

As shown in Chart 5, while exclusion of any or all of the three phosphates from the system markedly decreased the incorporation of ^{14}C labeled amino acids into protein, the addition of 4-nitroquinoline N-oxide produced merely a negligible inhibition. The lack of inhibiting effect here is in striking contrast to the case of intact cells.

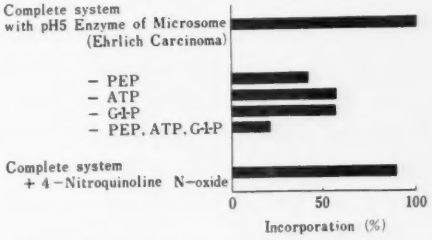


Chart 5. Effect of 4-Nitroquinoline N-Oxide on ^{14}C Amino Acid Incorporation into Protein.

Phosphorylation

The above described failure of 4-nitroquinoline N-oxide to inhibit the incorporation of ^{14}C-amino acids in a cell-free system raises the question of the effect of this substance on phosphorylation, and, in order to clarify this question, we attempted to trace the behavior of organic and inorganic phosphorus in the cell.

In this experiment four vessels were prepared, two of which containing 4-nitroquinoline N-oxide, 0.23 mg/m*l*. Other components of the reaction mixture in all the vessels were glucose, 4 mg/m*l*., and Ehrlich tumor cells, 12 wet weight volume percent, in final concentration in Krebs-Ringer bicarbonate solution, the total volume of the reaction mixture in each vessel being 30 m*l*.

After 10 minutes' incubation, 10 m*l* of the mixture was removed from each of the quinoline-added and control vessels, and necessary analysis was made of each immediately. ^{32}P (inorganic phosphate), 1 μc/m*l*, was added to all the vessels at this

time, and 15 minutes later 10 m*l* of the reaction mixture was again taken out from the two vessels and analysed. The two other vessels, one quinoline-added and one control, were treated similarly at the 45 minute period and reaction mixtures analysed.

The fractionation of inorganic (ortho) phosphorus and organic phosphorus compounds were carried out as follows: After the incubation, the cells were collected by centrifugation and washed three times with 10 m*l* of ice-cold 0.9 percent NaCl to remove the inorganic phosphorus contained in the medium. Washed cells were extracted twice with 0.5N perchloric acid, the extract amounting to 5 m*l* in total. An alliquot of the extract was analysed immediately for inorganic (ortho) phosphorus after the method of Fiske and Subbarow (11). The remainder of the extract was treated to remove ortho phosphorus according to the method of Martin and Doty (12). The aqueous phase, which contained organic phosphorus compounds, was separated and hydrolysed at 170°C for 3 hours. The phosphorus content in the hydrolysate was analysed by the same method as above.

Table 1, which represents the results, shows that inorganic phosphorus is decreased when the quinoline is added, more than in control. The increase of acid soluble organic phosphorus is seen almost equally in the two cases, but at an early period, i. e., within 30 minutes after the cells were allowed to contact quinoline, when the glycolytic inhibition is not yet evident, the increase in the quinoline-added mixture is greater than in control.

Table 1. The Amounts of and ^{32}P-incorporation into Inorganic and Acid Soluble Organic phosphates.

	Inorg. P			Acid soluble org. P		
	Total cpm	Total wt. (γ)	Specific activity	Total cpm	Total wt. (γ)	Specific activity
0 time		105.0			192.0	
Control 10 min.		63.3			225.0	
Control 25 min.	68400	49.6	1378	132000	247.0	535
Control 45 min.		64.3			223.0	
Control 60 min.	79600	42.5	1872	94600	221.0	428
+ 4-Nitroquinoline N-oxide. 10 min.		50.8			213.0	
+ 4-Nitroquinoline N-oxide. 25 min.	46400	58.9	788	192000	221.0	580
+ 4-Nitroquinoline N-oxide. 45 min.		37.0			234.0	
+ 4-Nitroquinoline N-oxide. 60 min.	16600	33.1	501	69100	205.0	337

The inhibition by the quinoline of ^{32}P incorporation into RNA-P, DNA-P, lipid-P, and protein-P was of about the same order of magnitude, as already stated, the inhibition amounting to 50 percent even at an early period, and reaching a marked

degree from the 30 minute period on, after the cells contacted the quinoline, when glycolytic inhibition first becomes evident. See Tables 2 and 3.

Table 2. The Amounts of and ^{32}P-incorporation into Nucleic Acids

	RNA			DNA		
	Total cpm	Total wt. (γ)	Specific activity	Total cpm	Total wt. (γ)	Specific activity
0 time		47.5			54.0	
Control 10 min.		42.0			66.0	
Control 25 min.	1491	43.5	34.3	4660	48.1	96.9
Control 45 min.		45.0			57.9	
Control 60 min.	1651	45.6	36.2	4120	62.9	65.4
+ 4-Nitroquinoline N-oxide. 10 min.		41.0			66.5	
+ 4-Nitroquinoline N-oxide. 25 min.	586	40.3	14.5	3065	87.0	35.2
+ 4-Nitroquinoline N-oxide. 45 min.		35.6			48.7	
+ 4-Nitroquinoline N-oxide. 60 min.	213	33.9	6.3	859	60.6	14.2

Table 3. The Amounts of and ^{32}P-incorporation into Lipid and Protein Phosphorus.

	Lipid-P			Protein-P		
	Total cpm	Total wt. (γ)	Specific activity	Total cpm	Total wt. (γ)	Specific activity
0 time		232.0			5.75	
Control 10 min.		224.0			5.75	
Control 25 min.	6860	232.0	29.6	5250	5.95	882
Control 45 min.		259.0			6.16	
Control 60 min.	7250	231.0	31.4	4440	5.42	820
+ 4-Nitroquinoline N-oxide. 10 min.		241.0			4.88	
+ 4-Nitroquinoline N-oxide. 25 min.	4900	228.0	21.5	4670	5.95	785
+ 4-Nitroquinoline N-oxide. 45 min.		222.0			4.68	
+ 4-Nitroquinoline N-oxide. 60 min.	1800	232.0	7.8	1060	3.91	271

These results appear to demonstrate that 4-nitroquinoline N-oxid does not inhibit phosphorylation, if it does not even increase it.

Analysis of Acid Soluble Organic Phosphorus Compounds

It is now important to analytically examine the acid soluble organic phosphorus in the above described experiment. For this purpose, paper chromatography and radioautography were utilized.

The organic phosphorus fraction obtained as in the preceding experiment was repeatedly extracted with 0.5N perchloric acid, and the combined extract was

adjusted to pH 6.0 with 4N KOH, using methyl red as indicator. Potassium perchlorate precipitate was removed by centrifugation and the supernatant was lyophilized, and the lyophilized material was extracted with 0.5 m*l* of ice-cold deionized water. 0.025 m*l* of this extract was spotted on paper (Tōyō Roshi, No. 51). The solvent systems used in the paper chromatography were MeOH, HCOOH, H_2O (16 : 3 : 1) mixture for acidic, and MeOH, conc. NH_4OH, H_2O (6 : 1 : 3) for basic solvents.

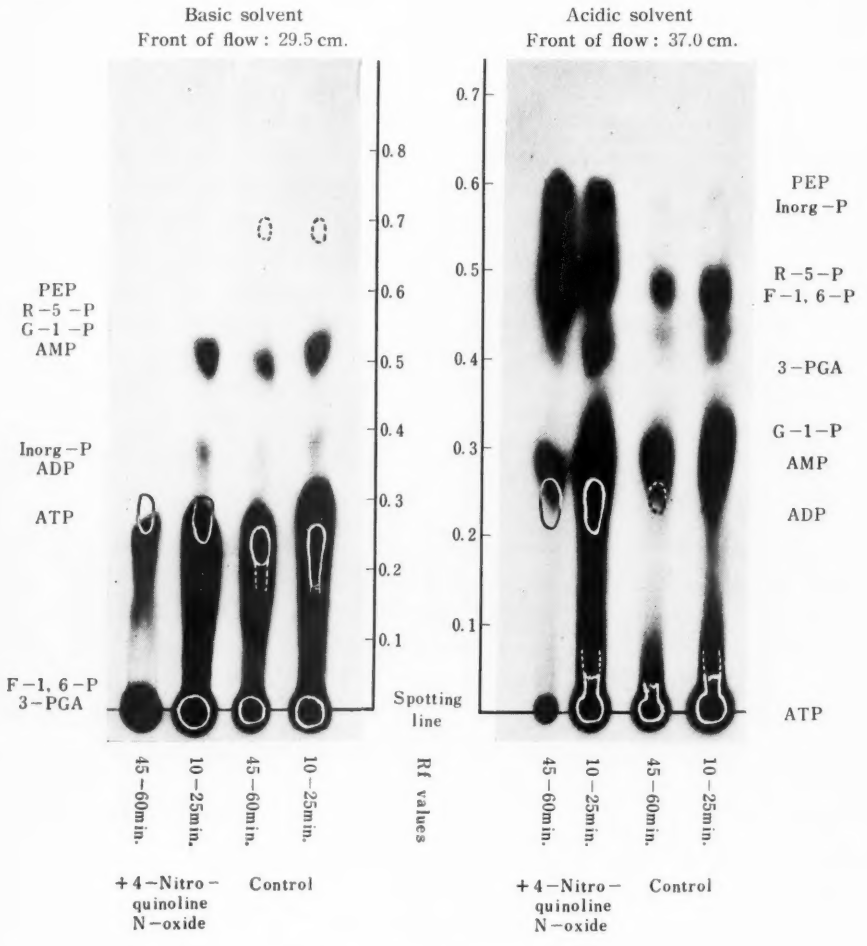


Fig. 1. One Dimensional Paper Radioautograms of ^{32}P-phosphorylated Compounds, with Ultraviolet Absorption Spots Superposed on the Radiogram.

The radioautogram of the paper chromatogram was obtained by keeping the paper in contact with an x-ray film for one week (Fig. 1).

Table 4 shows the Rf-values of standard samples of adenosine-monophosphate (AMP), adenosine-diphosphate (ADP), adenosine-triphosphate (ATP), glucose-1-phosphate (G-1-P), fructose-1, 6-diphosphate (F-1, 6-P), ribose-5-phosphate (R-5-P), 3-phosphoglyceric acid (3-PGA), phosphoenolpyruvate (PFP), and inorganic phosphate. Identification of the spots on radioautogram was made by referring to these standards. The position of the spot of nucleotide was established by the examination of ultraviolet absorption.

Table 4. The Rf-values on Paper Chromatograms of Standard Phosphorylated Compounds.

Compound	Acidic solvent*	Basic solvent**
AMP	0.27	0.48
ADP	0.20	0.43
ATP	0.00	0.26
G-1-P	0.34	0.55
F-1, 6-P	0.48	0.00
R-5-P	0.46	0.54
3-PGA	0.40	0.00
PEP	0.58	0.54
Inorg.-P	0.58	0.39

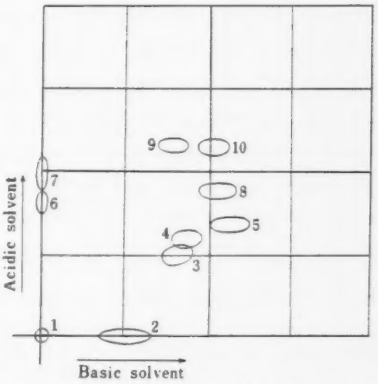
* MeOH, HCOOH, H_2O (16 : 3 : 1)

** MeOH, conc. NH_4OH, H_2O (6 : 1 : 3)

An examination of the radioautogram (Fig. 1) shows that the photosensitizing activities of the spots are generally much reduced in the quinoline-treated than in untreated samples, although the spot of F-1, 6-P and of 3-PGA are more strongly photosensitizing.

When compared by ultraviolet absorption, the spot of ATP was found to be exceedingly weak in the quinoline treated sample, while the spots of ADP and AMP were recognized more strongly than in the control sample.

In order to further facilitate the evaluation of the spots, two dimentional paper chromatography was carried out, using the same



1, Spotting point. 2, ATP 3, ADP 4, AMP 5, G-1-P 6, 3-P-G-A 7, F-1, 6-P 8, R-5-P 9, Inorg. P 10, PEP

Fig. 2. Two Dimendional Autoradiogram of ^{32}P-Incorporated Phosphorylated Compounds.

acidic and basic solvents as before. Fig. 2 shows the position of the spots of the eight acid soluble organic phosphorous compounds as they appear in the two dimentional paper chromatogram, which served as the standard for identification. By referring to this standard, the intensities of photosensitizing activities of the spots on radioautogram were compared between the quinoline treated and control samples. The results are expressed as in Table 5.

Table 5. Comparison of the Densities of the Spots.

Spot No.	Compound	Control 25 minutse	Control 60 minutes	4-Nitroquinoline N-oxide 35 minutes	4-Nitroquinoline N-oxide 60 minutes
(1)	Spotting Point				
(2)	ATP	+++	++	−	−
(3)	ADP	+	±	+	−
(4)	AMP				
(5)	Glucose-1-P	−	−	+	+
(6)	3-P-Glyceric acid	+	+	++	+++
(7)	Fructose-1, 6-P	+	+	++	+++
(8)	Ribose-5-P				
(9)	Inorg.-P	−	−	−	−
(10)	P-Enol-pyruvate	+	+	+	−

In an effort to render the above evaluations more quantitative, the spots of ATP, ADP, AMP, 3-PGA and F-1, 6-P were cut out of the chromatogram, and their radioactivities were measured by counts, using a conventional Geigar-Müller counter. The counts were corrected for self absorption. For ATP, ADP spots the intensities of ultraviolet absorption at 260 mμ were also measured and were taken into account. These results are presented in Tables 6 and 7.

It may be seen from these results that in the quinoline-treated sample ATP is very decidedly decreased both in amount and radioactivity, as evidenced by the ultraviolet absorption and count data. On the other hand, ADP and AMP appear to be increased in amounts, if judged by the ultraviolet absorption, and F-1, 6-P and 3-PGA to be very much increased when evaluated by the counts.

Table 6. The ^{32}P Counts of the Spots of ATP, 3-Phosphoglyceric Acid and Fructose-1, 6-diphosphate

	Incorporation period	ATP	3-Phosphoglyceric acid	Fructose-1, 6-diphosphate
Control	10～25 min.	2897	432	266
	45～60 min.	1397	522	348
+ 4-Nitroquinoline N-oxide	10～25 min.	2156	742	784
	45～60 min.	197	988	1095

Table 7. The Extinction at 260 mμ of the Extract of the Spot of ATP, and ADP plus AMP.

	Incubation time	ATP	ADP plus AMP
Control	25 min.	0.630	0.040
	60 min.	0.410	0.060
+ 4-Nitroquinoline N-oxide	25 min.	0.380	0.100
	60 min.	0.120	0.140

Each spot was extracted with 3 ml of 0.1 N HCl and its extinction was measured by Beckman DK-2 spectrophotometer.

Discussion

Experimental results described above, and deductions that can be made from them, are not without interest in the elucidation of the mode of biochemical action of 4-nitroquinoline N-oxide.

The fact that the inhibition by this quinoline of ^{32}P incorporation into nucleic acids as well as of ^{14}C-amino acid incorporation into protein takes place preceding the onset of glycolytic inhibition should be considered in connection with the effect of the quinoline on a cell-free system, which does not involve glycolysis. The microsome and pH 5 enzyme fractions from cancer cells, with the aid of PEP, ATP and G1P, readily permit the incorporation of ^{14}C-amino acids into protein, but the incorporation here was found not to be inhibited by 4-nitroquinoline N-oxide. Paralleling this result, inorganic and organic phosphorus were also found to behave in the same way in the quinoline-treated and untreated cells, apparently showing that the process of phosphorylation is not disturbed.

It was through the paper chromatographic analysis of acid soluble organic phosphorous compounds that marked increase of hexosediphosphate and triosephosphate (as evidenced by ^{32}P retention) and extrem decrease of adenosinetriphosphate were definitely established. The suppression of ATP production was observable very early in the progress of the reaction.

In glycolytic system ATP is required as a co-factore before the production of F-1, 6-P and triosephospate also. How does it happen, then, that incorporation and retention of ^{32}P in F-1, 6-P and triosephosphate goes on, despite the greatly reduced ^{32}P incorporation into and amount of ATP due to the quinoline effect? To explain this, it seems reasonable to postulate that the ATP in the cells acquires the degree of specific activity close to that of the control immediately after ^{32}P is added and that this labeled γP of ATP is transferred to F-1, 6-P and becomes accumulated there as the subsequent reactions are interrupted. It is known that γP of ATP is exchangeable with inorganic P (16) (17). This mechanism is supported

by the fact that although the absolute amount of ATP is greatly reduced, its specific activity is not decreased as markedly by the action of the quinoline.

From these considerations, it may be possible to conclude that the early inhibition of ^{32}P incorporation into nucleic acid and the labeled amino acid incorporation into protein by 4-nitroquinoline N-oxide may be due to the suppression of ATP production

Next to be considered is the possible mechanism whereby 4-nitroquinoline N-oxide inhibits glycolysis and suppresses the production of ATP. According to our paper chromatographic findings, among the various phosphorylated intermediates of the glycolysis system, F-1, 6-P and triosephosphate are accumulated in the cell, while phosphoenolpyruvate is decreased. From these circumstances it is possible to suggest that the glycolytic process is blocked at somewhere after the triosephosphate stage and certainly before the phosphoenolpyruvate stage. The fact that in the cell-free system the incorporation of amino acid into protein is not inhibited by the quinoline also suppots this suggestion that the glycolysis of intact cells is inhibited at a stage before phosphoenolpyruvate becomes involved. Since in anaerobic glycolysis the production of ATP occurs at a stage between 1, 3-diphosphoglycerate and phosphoenolpyruvate, the ATP production would be expected to be reduced as a natural consequence if the inhibition of glycolysis did take place at the stage of triosephosphate.

It may be pointed out that triosephophate dehydrogenase is an enzyme known to be especially sensitive to chemicals damaging to sulphydryl group (14). It is also to be noted that the glycolytic inhibition by monoiodoacetic acid is generally accepted to be due to the inhibition of this enzyme. These considerations permit the inference that a similar mechanism may be at work in the case of 4-nitroquinoline N-oxide, which is very highly reactive to sulphydryl group. We hope to elucidate this point by our further experiment.

Summary and Conclusion

Confirmation was obtained of the marked inhibition by 4-nitroquinoline N-oxide of glycolysis and of the ^{32}P incorporation into nucleic acids and ^{14}C-labeled amino acids into protein of cancer cells, but it was discovered that the inhibition of the incorporation takes place before the glycolytic inhibition becomes evident. This led to the examination of the incorporation in a cell-free system, in which glycolysis is not involved, and it was established that 4-nitroquinoline N-oxide does not inhibit the incorporation, in striking contrast to the case of intact cells system. It was also found that the quinoline produces no notable effect on phosphorylation. However, by analysis of acid soluble organic phosphates by paper chromatographic and radioautographic means, as well as through ultraviolet absorption, it was

demonstrated that ATP is very decidedly decreased in the quinoline-treated samples, while F-1, 6-P and 3-PGA are increased.

From these and other findings, it was tentatively concluded that 4-nitroquinoline N-oxide suppresses ATP production, which may account for the inhibition of nucleic acid and protein synthesis. As to the glycolytic inhibition the fact that there is a reduction of phosphoenolpyruvate under the quinoline influence suggested that this latter may block the process by damaging triosephosphate dehydrogenase.

References

1. Sakai, S., Minoda, K., Saito, G,. Akagi, S., and Fukuoka, F., Gann, **46**: 605 (1955).
2. Nakahara, W., Fukuoka, F., and Sugimura, T., Gann, **48**: 124 (1957).
   Nakahara, W., Fukuoka, F., and Sakai, S., Gann, **49**: 33 (1958)
3. Nakahara, W., and Fukuoka, F., Gann, **50**: 1 (1959).
4. Fukuoka, F., Sugimura, T., and Sakai, S., Gann, **48**: 65 (1957).
5. Fukuoka, F., and Naora, H., Gann, **48**: 271 (1957).
6. Wada, T., Nomura, M., Mitsui, H., Maruo, B., Tamiya, H., and Akabori, S., J. Gen. Appl. Microbiol., **1**: 142 (1955).
7. Schmidt, G., and Thanhauser, S. J., J. Biol. Chem., **161**: 83 (1945).
8. Schneider, W. C., J. Biol. Chem., **161**: 293 (1946).
9. Littlefield, J. W., and Keller, E. B., J. Biol. Chem., **224**: 13 (1957).
10. Lowry, D. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., **193**: 265 (1951).
11. Fiske, C. H., and Subbarow, Y., J. Biol. Chem., **66**: 375 (1925).
12. Martin, J. B., and Doty, J. H., Anal, Chem., **21**: 965 (1949).
13. Bandurdki, R. S., and Axelrod, B., J. Biol. Chem., **193**: 405 (1951).
14. Barron, E. S., Advanc., in Enzym., **11**: 201 (1951).
15. Wade, H. E., and Morgan, D. M., Nature, **171**: 529 (1953).
16. Krebs, H. A., Ruffo, A., Johnson, M., Eggleston, L. V., and Hemms, R., Biochem. J., **54**: 107 (1953).
17. Lee, K. H., and Eiler, J. J., J. Biol. Chem., **203**:705 (1953).

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THE TOXIC TUMOR FACTOR DEPRESSING DPN SYNTHESIS IN TUMOR-BEARING ANIMALS

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INTRODUCTION

Since Nakahara and Fukuoka (1) demonstrated a liver catalase depressing factor, the so-called toxohormone, in the malignant tissues, its role in depressing the liver catalase activity in tumor-bearing animals has been well established. The work to elucidate the chemical nature of toxohormone has been continued in this laboratory. Recently Waravdekar (2) reported that the DPN synthesis activity in the liver of tumor-bearing animal was lower than that of the control, and suggested the existence of humoral factor in the tissues and blood of animals bearing progressively growing tumors, which decreased the DPN synthesis enzyme in tissues. The apparent similarity observed between the mode of depressing the DPN synthesizing enzyme in tumor-bearing animals and that in the case of liver catalase depression greatly attracted our attention, and it was considered necessary to elucidate the nature of the inhibitory factor of DPN synthesis in correlation with that of toxohormone.

For this purpose the DPN levels in the liver of tumor-bearing animals and the decreasing rates of DPN synthesizing activities *in vivo* as well as *in vitro* were examined in comparison with those of normal and pregnant ones. Then the turnover rates of DPN in the liver of normal and tumor-bearing rats were compared by ^{32}P incorporation rates into the nucleotides, in nicotinamide administered and untreated rats.

It was found that the injection of tumor tissue homogenate caused a remarkable depression of DPN synthesis *in vivo*. As one trial of extraction of the active principle, raw toxohormone was prepared according to the original method of

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The following abbreviations are used throughout the present paper: AMP, ADP, ATP=adenosine mono-, di- and triphosphates; similarly UMP, UDP, UTP (uridine); GMP, GDP (guanosine); DPN and TPN=di- and triphosphopyridine nucleotide; IMP=inosine monophosphate; UDPAG=uridine diphosphate N-acetylglucosamine; UDPG=uridine diphosphate glucose; NMN=nicotinamide mononucleotide; TCA=trichloroacetic acid; PCA=perchloric acid.

Nakahara and Fukuoka (1) and tested for this activity. Fortunately this trial was successful. The more purified toxohormone preparations (3) prepared by several means were then tested and found also to be effective *in vivo*.

The effects *in vitro* of the homogenates of the liver of tumor-bearing animal and tumor tissue itself on the DPN synthesis activity of normal liver homogenates were also examined, but entirely failed to confirm the inhibotory action observed by Waravdekar (2).

Methods and Materials

Determinations of DPN and DPNH: The determination of DPN amount in the animal liver was carried out by the method of Jedikin et al. (4). Oxidized DPN (DPN^+) in the liver was extracted from the tissue by boiling for 1 minute after homogenization with 9 volumes of pH 5.4, 0.015M phosphate buffer followed by removal of the denatured protein. Extraction of reduced DPN (DPNH) in the liver was carried out exactly in the same manner, except that the phosphate buffer of pH 8.7 was used.

The amount of DPN was measured by the increase of absorption at 340 mμ after the addition of alcohol dehydrogenase and ethanol in pH 9.0, 1/30M pyrophosphate buffer. The amount of DPNH was measured by the reduction of absorption at 340 mμ with the aid of alcohol dehydrogenase and acetaldehyde in pH 7.0, 1/30M phosphate buffer.

Measurement of DPN increase with nicotinamide administration: The increase of DPN amount in the liver was measured 8 hours after injection of nicotinamide intraperitoneally 500 mg/kg animal body weight (5) and the fluctuation of nucleotides in the liver after nicotinamide administration was pursued after 4 hours of the injection.

Separation of acid-soluble phosphates in the liver: Rats were injected with radioactive phosphate (pH 7.0, 200 μc in 1 m*l* of 40 μM inorganic phosphate solution) at 75 μc per 100 g body weight (6). 15 minutes after the injection, livers wer quickly excised, frozen in the dry-ice ether and weighed out approximately 15 g was equally from two rats. The liver was homogenized in glass homogenizer with 2 volumes of 0.6 N perchloric acid, the protein was centrifuged and then resuspended in 2 volumes of 0.2 N PCA. The initial extract and the washings were combined and neutralized with 5 N KOH. The supernatant was condensed to 5 m*l* by means of lyophilization, and then was charged on the ion exchange column of Dowex-1, X-10 (fromate form, 15 X 1.0 cm). The separation of compounds by ion exchange chromatography was carried out by gradient elution procedure of formate system as described by Hurlbert et al. (7), using 250 m*l* mixing volume and changing the eluents: 100 m*l* 1N formic acid, 250 m*l* 0.4 N formic acid, 325 m*l* 0.2N ammonium

formate in 4 N formic acid, 250 m*l* 0.4 N ammonium formate in 4 N formic acid and 250 m*l* 1 N ammonium formate in 4 N formic acid, in the order named. The fractions of approximately 5 ml were collected at rate of 0.4 m*l* per minute and then were analysed as follows :

a) ***Ultraviolet absorption*** : The optical density of the fractions of chromatograms were read at 245, 260, 275 and 290 mμ in Beckman model DU spectrophotometer in 1 cm quartz cells with 0.1 N HCl as the blank, and then various nucleotides in each fraction were judged by the ratio of the readings at 275 and 260 mμ (8).

b) ***Phosphate*** : Inorganic phosphate and total phosphate were determined by Allen's method (9).

c) ***Measurement of radioactive phosphate*** : Each 0.2 m*l* of fractions were taken in the sample plates and after dried, the radioactivities were measured by G. M. counter, and their specific acitivities (cpm/γ total P) of nucleotide were calculated.

Measurement of DPN synthesizing enzyme activity : The assay of the activity of DPN synthesizing enzyme was carried out according to the method of Kornberg (10), except fluoride was introduced additionally. The reaction mixture (pH 7.5, final volume 1.0 m*l*) was made up at 0° and contained the following components : 0.002 M ATP, 0.0025 M NMN, 0.05 M glycylglycine–KOH buffer pH 7.4, 0.015 M $MgCl_2$, 0.2 M nicotinamide 0.05 M KF and the enzyme (10% liver homogenate in water, 0.1 m*l*). The mixture was then shaken in water bath at 38° and the reaction was stopped at 1 hour by the addition of 1.0 m*l* of cold 10% TCA, and then DPN content was measured.

The fluoride addition in the reaction was used in order to improve the proportionality of the synthesized DPN amount and incubation period or enzyme amount, as shown in Fig. 1.

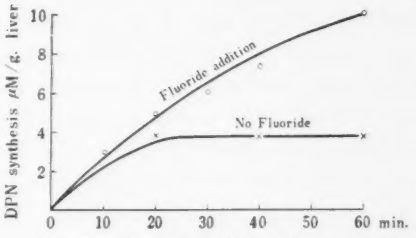

Fig. 1. The effect of fluoride on DPN synthesizing system.

Materials : ATP (disodium salt) and DPN (90% purity) was obtained from the

Table 1. Result of analysis of NMN.

	Method	Ratio
Nicotinamide-N substituted body	Flurometry (18)	1.0
	Hydrosulfide reduction. (19)	1.0
Ribose	Meijbaum's method.	1.0
Organic phosphate	Allen's method. (9)	1.1
Inorganic phosphate		0.0

Sigma Co., Ltd., NMN was prepared by enzymatic hydrolysis of DPN according to the method of Kornberg (11, 12) in our laboratory, and the results of its analysis were shown in Table 1.

Crystalline alcohol dehydrogenase was isolated from the dry yeast of the Oriental Co., Ltd., by the procedure of Racker (13).

Tumors: The transplantable tumors of mice used in this experiment were Sarcoma 180 (S 180), Nakahara-Fukuoka carcinoma (NF-C), and spontaneous dba mammary carcinoma in dba mice. The tumor of rat used was Rhodamine fibrosarcoma (Umeda).

Results

In vivo synthesis of DPN: There were presented in Fig. 2 the levels of DPN and the increase of DPN in the liver after nicotinamide administration in normal, tumor-bearing and toxohormone injected animals. It was shown that about 3 weeks after the tumor transplantation (S-180, NF-C and Rhod. Sarcoma), the DPN increase by nicotinamide administration was 30, 25 and 30 per cent lower, respectively, than the control, but the DPN levels showed no difference between the tumor-bearing animals and the control. The DPN synthesizing capacity in the liver of the pregnant mice was revealed not to decrease below that of the control. The DPN increase in the liver by nicotinamide administration was inhibited 24 hours after the injection of Rhodamine sarcoma homogenate and raw toxohormone to mice in the doses sufficient to depress the liver catalase activity as far as the level of the tumor-bearing animals is concerned. The catalase activities in the all groups were shown in Fig. 3.

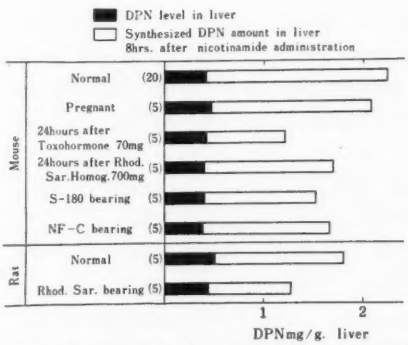

Fig. 2. DPN level and synthesized DPN amount after nicotinamide administration. Figures in the parenthesis are the numbers of animals in the groups.

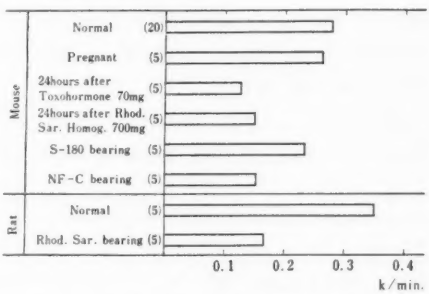

Fig. 3. The mean of liver catalase activities of each group.

DPN synthesis enzyme activity: Liver of tumor-bearing mice was revealed to show lower activity of DPN synthesizing enzyme than the control, as presented

in Fig.4, that is, activity of NF-C bearing mice decreased 50 percent, and that of dba mice with transplanted mammary carcinoma lowered 58 percent of the control. The activities of DPN synthesizing enzyme and catalase of the livers from mice, 24 hours after the injection of raw and purified toxohormone fractions were also depressed to the level comparable with that of tumor-bearing animals, as illutrated in Fig. 5. The activities of DPN synthesizing enzymes of the mouse livers, 24 hours after the injection of Rhodamine sarcoma homogenate and raw toxohormone, were lowered to 20 and 30 percent of that of the control. The injection of toxohormone and its fractions purified by DEAE-cellulose column chromatography (3) to mice depressed the activities of DPN synthesizing enzyme and catalase in the livers almost equivalently, but injection of another fraction of toxohormone obtained by DEAE-cellulose column chromatography of the methanolacetic acid extract of the raw toxohormone (14), exhibited less effect on the DPN synthesizing activity than on the catalase.

In vitro effect of tumor homogenate and toxohormone on the DPN synthesizing enzyme: First, the homogenate of normal mouse liver, liver of dba mammary carcinoma-bearing mouse and mammary cacinoma tissue were assayed separatery, and then the effect of the combination of two different homogenates, that is, normal and tumor-bearing mouse livers or tumor tissue, were examined. But as illustrated in Fig. 6, the results of combination tests were nearly additive within the limits of

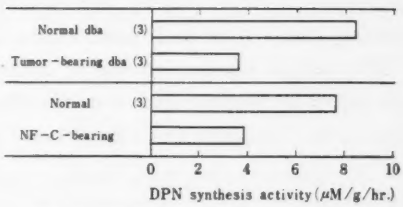

Fig. 4. DPN synthesis activities of normal and tumor bearing mice.
(Figures in the parenthesis are the numbers of animals in the groups.)

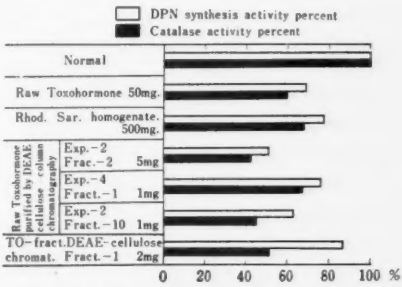
DPN synthesis activity percent
Catalase activity percent
Normal
Raw Toxohormone 50mg.
Rhod. Sar. homogenate. 500mg.
Raw Toxohormone purified by DEAE cellulose column chromatography
Exp.-2 Frac.-2 5mg
Exp.-4 Fract.-1 1mg
Exp.-2 Fract.-10 1mg
TO-fract. DEAE-cellulose chromat. Fract.-1 2mg
0 20 40 60 80 100 %

Fig. 5. The effect of tumor homogenate and toxohormone fractions on liver DPN synthesis activity and catalase activity *in vivo*.

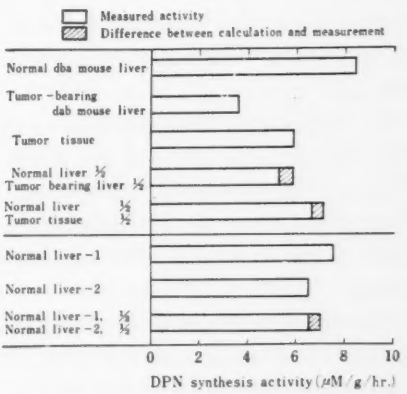
Measured activity
Difference between calculation and measurement
Normal dba mouse liver
Tumor-bearing dab mouse liver
Tumor tissue
Normal liver ½ Tumor bearing liver ½
Normal liver ½ Tumor tissue ½
Normal liver-1
Normal liver-2
Normal liver-1, ½ Normal liver-2, ½
0 2 4 6 8 10
DPN synthesis activity (μM/g/hr.)

Fig. 6. The effects of tumor-bearing mouse liver and tumor tissue extract on DPN synthesis of normal mouse liver *in vitro*.

experimental error, and so there was demonstrated no inhibitory action in tumor-bearing mouse liver and tumor tissue *in vitro*.

The relative amounts of and ^{32}P incorporations into the acid soluble nucleotides in the livers of normal and tumor-bearing rats: The absorption at 260 mμ of each nucleotide calculated per 1 g of liver of each group was shwon in Table 2. The

Table 2. Absorption at 260 mμ of acid-soluble nucleotides from 1 g of rat liver.

	Normal	Rhod. sar. bearing rat	4 hours after nicotinamide administration	
			Normal	Phod. sar. bearing rat
DPN	7.44	7.36	25.36	22.07
TPN	2.07	2.81	2.32	2.17
AMP	17.18	20.70	25.30	25.10
ADP	12.42	15.40	11.70	7.21
ATP	6.34	7.38	3.72	1.82
ADP-X	4.08	5.13	4.54	5.26
IMP	2.97	3.36	4.37	4.86
UMP	7.79	5.46	7.72	7.90
UDP	3.67	3.89	5.61	3.26
UDPAG	3.48	2.98	3.85	3.72
UDPG	3.12	4.72	2.42	3.21
GMP	2.52	3.34	4.82	4.11
X	—	—	1.53	1.68
Total	73.18	82.64	103.25	92.37

total extinction at 260 mμ of the perchloric acid extract from the liver of tumor-bearing rat was higher than that of the control, especially increased being the adenine nucleotides such as AMP, ADP and ATP. IMP, GMP and UDPG of tumor-bearing rat also increased slightly but UMP and UDPAG decreased below those of the control.

By nicotinamide administration to the normal rat, the DPN amount in the liver was multiplied several fold and AMP, IMP, ADP-X UDP and GMP were decreased compared to the control. Comparing the response of tumor-bearing and normal rats to the nicotinamide administration, it was demonstrated that the decrease of ATP in the liver from tumor-bearing rat was more exaggerated than in the normal liver.

Relative specific activities (RSA) of each of the nucleotides in the liver were presented in Table 3. The RSA of DPN was the lowest among the RSA of identified nucleotides, and the RSA of DPN in the liver of tumor-bearing rats was lower than that of normal ones. Nicotinamide administration decreased the RSA of DPN but in this case that of tumor-bearing rats was also lower than normal. Turning to the other nucleotides than DPN, the RSA of adenine nucleotides was

Table 3. Relative specific activies of acid-soluble nucleotides in the rat liver.

	Normal	Rhod. sar. bearing rat	4 hours after nicotinamide administration	
			Normal	Rhod. sar. bearing rat
DPN	.010	.0081	.0086	.0077
AMP	.141	.115	.191	.154
ADP	.648	.523	.675	.495
ATP	.764	.672	.626	.730
ADP-X	.079	.023	.053	.045
UMP	.064	.105	.040	.046
UDP	.331	.343	.131	.244
UDPAG	.167	.177	.210	.220
UDPG	.472	.385	.758	.338
GDP	.189	.249	.220	.382

lower in tumor-bearing than in normal rats and this relation was also maintained after nicotinamide administration.

Discussion

The fact that the tumor transplantaion depresses the DPN synthesizing activity in the liver, as reported by Waravdekar (2), was confirmed by the ***in vitro*** assay experiment and also by ***in vivo*** experiments, in which the DPN increase following nicotinamide administration was compared. Narurkar (15) reported consistent results with those described here by the experiment of nicotinamide administration to the tumor-bearing animals, but he performed only ***in vivo*** synthesis experiment, and suggested the possibility that adenine deficiency may be produced in the animal by the rapid growth of tumor tissue. This suggestion on the mode of depressing DPN synthesis cannot be acceppted, because of our findings that DPN synthesizing enzyme itself was reduced in the liver as revealed by ***in vitro*** tests, and that pregnant mice showed no decreased rate of DPN synthesis at all after nicotinamide administration. It is strongly contradicted also by our findings that the injection of tumor homogenate as well as raw toxohormone preparation caused the depression. The DPN synthesis ***in vivo*** (response to nicotinamide) and ***in vitro*** (synthesis activity from ATP and NMN) supported strongly the toxin theory.

The purification of the active principle for depressing DPN synthesis was not carried out in this study, but in the course of the purification of toxohormone as assayable by the catalase depressing activity, of which details will be published later in this journal, several fractions of different purities were tested and revealed to be active ***in vivo*** in depressing DPN synthesis in degrees corresponding to catalase depression. But one fraction obtained by DEAE-cellulose column chromatography of methanol-acetic acid extraction from raw toxohormone exhibited lower

activity in depressing DPN synthesis than in depressing catalase. It seems doubtful that one and the same toxic substance is responsible for the both actions, but the decisive solution of this problem must be postponed until higher purification should be attained. About the nature of this active substance, it must be noted that these partially purified toxohormone fractions consisted almost entirely of proteins.

Although our finding supported the toxic theory proposed by Waravdekar et al., it must be emphasized that neither toxohormone nor the homogenates of tumor and the liver of tumor-bearing animal exhibited inhibitory action on DPN synthesis enzyme *in vitro*, contrary to their observations. For the reason of this descrepancy in the two laboratories, our modification of enzyme system by adding fluoride in in the reaction mixture could be considered. Kornberg's assay system (10) which was employed exactly by Waravdekar et al. was not satisfactory for so crude an enzyme source as liver homogenate to attain a straight action-time relation.

From the experiments of ^{32}P incorporation into each acid soluble nucleotide in the liver, it was demonstrated that the relative specific activity of DPN was especially low, consistent with such other data as reported by Brum et all. (6), and that relative specific activities of DPN from tumor-bearing rats were lower, either in the case of no pretreatment or after nicotinamide administration, than the corresponding controls. The first results must explain the observation that the depression of DPN level is brought about in the liver long after the appearance of decrease in DPN synthesis activity following transplantation of slowly growing tumor. But, as will be reported in another place by the authors (17), the capacity of DPN synthesis enzyme is far over the need to maintain DPN level, so the decrease of DPN level must be delayed until after that of DPN synthesis activity. After nicotinamide administration, in spite of the enormous increase of DPN, there was demonstrated no decrease of adenine nucleotides AMP, ADP and ATP, and only decrease in the ratio of ATP and AMP. It was therefore suggested that the adenine moiety in the newly synthesized DPN must be derived from another metabolic pool than free nucleotides, for example, RNA, as discussed by Shuster et al. (16). The differences of the nucleotides patterns observed between normal and tumor-bearing rats will be discussed elsewhee (17).

Summary

1) DPN synthesis activities in the livers of tumor-bearing animals showed lower level than that of the control.

2) Tumor homogenate, toxohormone and its partially purified fractions inhibited *in vivo* the DPN synthesis activity of the liver, but this effect was not confirmed *in vitro*.

3) The rate of ^{32}P incorporation was the lowest into DPN among the acid soluble nucleotides and the relative specific activity of DPN in the liver of tumor-bearing rat was a little lower than that of normal one.

Our hearty thanks are due to Dr. Waro Nakahara for helpful suggestions and criticism.

References

1. Nakahara, W., and Fukuoka, F., Gann, **40**: 45, (1949); Japan Med. J., **1**: 271, (1948).
2. Waravdekar, V. S., and Powes, O. H., J. Nat. Cancer Inst., **18**: 145, (1957).
3. Ohashi, M., and Ono, T., Gann: Proceedings of the 17th Meeting of Cancer Association. (1959).
4. Jedeikin, L. A., and Weinhouse, S., J. Biol. Chem., **213**: 271, (1955).
5. Kaplan, N. O., Goldin, A., Humphres, S. R., Ciotti, M. M., and Stalzenbach, F. M., J. Biol. Chem., **219**: 287, (1956).
6. Brumm, A. F., Potter, V. R., and Siekevity, P., J. Biol. Chem., **220**:713, (1956)
7. Hurlbert, R. B., Schmitz, H., Brumm, A. F., and Potter, V. R., J. Biol. Chem., **209**: 23, (1954).
8. Schmitz, H., Hurlbert, R. B., and Potter, V. R., J. Biol. Chem., **209**: 41, (1954).
9. Alien, R. J. L., Biochem. J., **34**: 858, (1940).
10. Kornberg, A., J. Biol. Chem., **182**: 779, (1950).
11. Kornberg, A., J. Biol. Chem., **182**: 763, (1950).
12. Shemin, D. ed., Biochemical Preparation, **5**: 55, (1957).
13. Racker, E., J. Biol. Chem., **183**: 313, (1950).
14. Ono, T., Sugimura, T., and Umeda, M., Gann., **46**: 617, (1955).
15. Narurkar, M. V., Kumta, V. S., and Sahasrabudhe, M. B., Brit. J. Cancer. **11**: 482. (1957).
16. Shuster, L., Langan, T. A., Kaplar, N. O., and Goldin, A., Naure, **182**: 512, (1958).
17. Ono, T., and Tomaru, T., in preparation,
18. Jevitas, N., Robinson, J., Rosen, F., Huff, J. W., and Perlzweig, W. A., J. Biol. Chem., **167**: 169, (1947)
19. Lepage, G. A., J. Biol. Chem., **168**: 627, (1947)
20. Meijbaum, W., Z. Physiol. Chem., **258**: 117, (1939)

[GANN, 50, 47~50; March, 1959]

CHEMOTHERAPEUTIC TESTS USING PULMONARY TUMOR FORMATION BY INTRAVENOUS INJECTIONS OF CANCER CELLS

(Plate II)

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The significance of secondary tumors in chemotherapeutic study is too obvious to require any comment, and yet, with the notable exception of Haruo Sato's (1) experiments using certain strains of mouse hepatomas, there has been few attempts reported in literature to utilize metastatic growths for the purpose of such studies. The reason for this paucity is undoubtedly due to the fact that most of the transplantable animal tumors usually do not metastasize. Baserga and Baum (2) reported that a transplantable mouse tumor, which is non-metastasizing as a rule, produces secondary tumors in the lung if it was subcutaneously transplanted into the tail. Unfortunately, their findings were not reproduced in our attempts to do so, using Ehrlich carcinoma. We found, however, that when the same carcinoma in the ascites form was injected intravenously, there readily arose multiple tumors in the lung, closely resembling the pulmonary metastases.

In the present paper we briefly describe the pulmonary tumors produced by intravenous injections of Ehrlich ascites carcinoma, and report on our attempts to test the effect of some of the known anti-cancer chemical agents on the formation of these artificial "metastases". It may be pointed out that the two methods currently in much use in experimental chemotherapeutic studies are attacking tumor cells in highly unnatural situations; treatment of ascites tumors by intraperitoneal injections is little short of test-tube experiments, while treatment of subcutaneous tumor grafts only one or two days after transplantation is to attack the tumor cells which are at a great disadvantage, not paralleled by any natural condition. The use of the artificial "metastasis" formation is far superior to either of these methods in that here we are testing the ability of chemical agents to prevent the tumor cells in the circulating blood from establishing themselves in the pulmonary tissue, very much in the manner of natural processes of metastasis formation. Any chemotherapeutic agent capable of markedly affecting such a process would be of great value in clinical practice.

Pulmonary Tumor Formation by Intravenous Injections of Ehrlich Ascites Carcinoma Cells

For the injections of Ehrlich carcinoma into the caudal vein to produce tumor

nodules in the lung of normal mice, fresh tumor ascites, aseptically aspirated 7 days after the intraperitoneal inoculation well serves. The amount of ascites to be injected need not be more than 0.02 cc. This amount of ascites usually contains about 800,000 cancer cells.

The early microscopical findings in the lung consist of disseminated capillary embolism of the tumor cells (Plate II, fig. 1), and the formation of small tumor nodules can be demonstrated by the 4th day (Plate II, fig. 2). By the 7th day multiple small nodules can be seen in gross (Pl. II, fig. 3), and these usually grow into extensive and well developed tumors by 14th day. Of organs other than the lung, heart and less frequently brain show tumor nodules, but liver and spleen do not seem to become involved.

The regularity with which the pulmonary tumors are produced by this method seems to depend upon the number of tumor cells actually delivered into the blood stream, which, according to our estimation, may be about 800,000 or more. Sometimes, though infrequently, assumed "perfect injections" fail to give rise to lung nodules, and we look upon such cases as being due to some technical failure. It is not always easy to be absolutely sure of the exact amount of ascites injected into the tail vein in mice. This difficulty can be overcome only through experience and increased skill.

Effect of Selected Anti-cancer Agents

1. In the first set of experiments we tested effect of 6-carboxy-4-nitroquinoline N-oxide (3), methyl-bis (β-chlorethyl) amine N-oxide (nitromine), and mitomycine C (4) on the formation of pulmonary tumors. Normal mice were first injected intravenously each with 0.02 cc of Ehrlich ascites containing about 800,000 cells. They were divided into 2 groups of 5 mice each, one group to be treated and the other group to remain untreated for control. Chemical treatment was started 24 hours after the ascites injection. The amount of 6-carboxy-4-nitroquinoline N-oxide was 25 mg/kg, of nitromine 2 mg/kg, and of mitomycine C 1 mg/kg per injection per mouse. The substances were dissolved in physiological salt solution and were delivered by intraperitoneal injections every day for 7 consecutive days. These doses, approximately 1/5 of LD_{50} of each substance, were chosen as representing the effective amounts in the intraperitoneal treatment of the ascites form of the tumor. All the mice were killed 10 days after the ascites injection, and the lung findings were compared with the following results:

Five mice treated with 6-carboxy-4-nitroquinoline N-oxide all showed lung nodules, so also did the 5 controls. The size of the nodules seemed to be generally larger among controls than in treated mice, but the difference, if real, was not remarkable.

With nitromine, all the mice, 5 in number in both treated and control groups,

showed lung nodules, with scarcely any difference between the groups as to the size or number of the nodules.

In experiment with mitomycine C, only 2 of the 5 control, and 4 of the 5 treated mice showed the nodules. The very low percent of positive tumors in controls may indicate some unsuspected technical failure in this experiment, but it seems fair to regard the results as suggesting that the treatment did not markedly affect the formation of the pulmonary nodules.

2. In another experiment, effect of Hg-hematoporphyrin sodium (5) and of triethylene thiophosphoramide (Thio-TEPA) were tested in the same way as above. On this occassion, mice were killed 21 days after the intravenous tumor inoculation to compare the lung findings. The amount of Hg-hematoporphyrin sodium and Thio-TEPA was 4.6 mg/kg and 3.4 mg/kg per injection per mouse, respectively, delivered intraperitoneally in 7 consecutive daily doses beginning 24 hours after the tumor inoculation. The results were:

Tumor nodules in 2 of the 5 untreated control mice, and in 3 of the 5 mice in two groups treated either with Hg-hematoporphyrin sodium or with Thio-TEPA. The poor yield of lung tumors in this experiment was disappointing, but the results suggested that no marked effect may be expected from either of the two agents tested.

3. Finally, the effect of 6-carboxy-4-nitroquinoline N-oxide, mitomycine C, 8-azaguanine and 6-mercaptopurine were compared by survival tests as well as by the lung findings at autopsy, the general experimental conditions being the same as in the preceding.

With 6-carboxy-4-nitroquinoline N-oxide, at the same dosage level as before, the survival periods of 5 mice in the group were 12, 13, 17, 19 and 23 days, contrasted to 15, 18, 19, 23 and 38 days in the same number of untreated controls. At autopsy tumor nodules were found in all the mice in both groups.

With mitomycine C, also in the same dosage as before, 5 treated mice survived 18, 24, 51, 51 and 89 days, against 24, 44, 51, 67 and 86 days in 5 untreated controls. Correspondingly, no perceptible difference between the two groups was noted in the lung findings at autopsy, all the mice showing well developed lung tumors.

In experiment with azaguanine and 6-mercaptopurine, in doses of 308 mg/kg and 3.8 mg/kg per injection per mouse respectively, seven 8-azaguanine-treated mice survived 19, 20, 20, 25, 25, 25, 25 and 27 days, seven 6-mercaptopurine-treated mice, 22, 24, 25, 30, 37, 43 and 60 days, and the same number of untreated controls, 22, 22, 29, 30, 37, 44 and 60 days. At autopsy, all the mice showed well developed lung tumors, irrespective of the treatment.

Conclusion and Comments

While it is impossible to draw any final conclusion on the basis of the preliminary experiments using a small number of animals, the results described in this paper show that none of the chemicals tested, though known to inhibit at least some forms of tumor growths under other experimental conditions, produced any demonstrable effect on the production of pulmonary tumors by intravenous injections of Ehrlich ascites carcinoma. Perhaps better results might have been obtained if chemical treatments were made through the intraveous route, instead of by intraperitoneal injections, but this remains yet to be proved.

It seems justifiable to state that the process of pulmonary implantation and growth of blood borne cancer cells may be more difficult to suppress than the more conventional types of tumor transplantations, and that it offers a new challange to future studies in experimental cancer chemotherapy.

The authors wish to express their thanks to Dr. Waro Nakahara, Director of Cancer Institute, for his guidance, to Dr. Kunio Oota for examining our histological specimens, and to Dr. Susumu Obata for technically cooperating in a part of the experiments reported in this paper.

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Literature cited

1) Sato, H. Symposium on the Chemotherapy of Cancer, Tokyo, U.I.C.C. Abstracts : 77 (1957).

2) Baserga, R., and Baum, J. Cancer Research, **15,** 52 (1955).

3) Fukuoka, F., Sugimura, T., and Suzuki, S. Gann, **48,** 263 (1957).

4) Wakaki, S., Marumo, H., Tomika, K., Shimizu, Y., Kato, E., Kamada, H., Kudo, S., and Fujimoto, Y. Antibotics and Chemotherapy (Japan). **8,** 228 (1958).

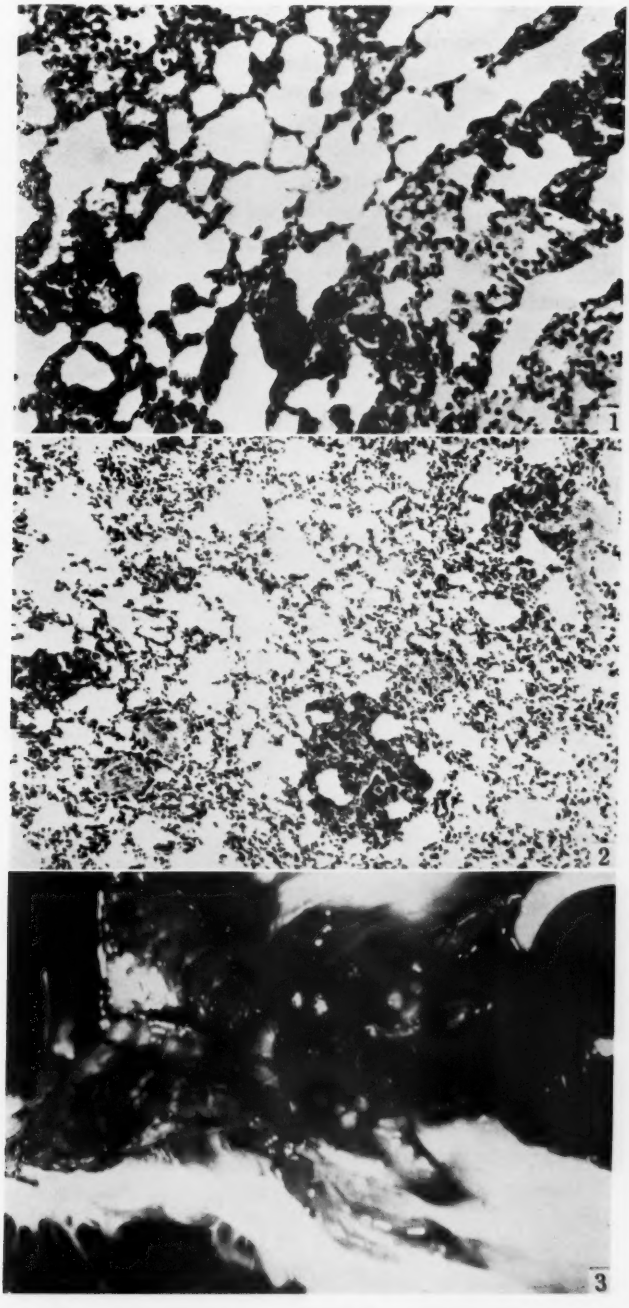
5) Iijima, N., Matsuura, K., Fujita, K., Aiba, T., Ukishima, H., and Kamish'rodo, K. Chemotherapy (Japan), **6,** 121 (1958).

Explanation of Figures (Plate II)

Fig 1. Embolism of tumor cells in the lung, 48 hours after an intravenous injection of Ehrlich ascites carcinoma.

Fig. 2. Small tumor nodule in the lung, 4 days after an intravenous injection of Ehrlich ascites carcinoma.

Fig. 3. A mouse 10 days after an intravenous injection of Ehrlich ascites carcinoma, showing multiple tumor nodules in the lung.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[GANN, 50, 51~56; March, 1959]

SOME CHEMICAL CHANGES IN THE LIVER OF RATS FED p-DIMETHYLAMINOAZOBENZENE. II

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The biochemical analysis of the process through which normal cells are transformed to cancer cells, has an important part in cancer research. Among the chemical changes occurring in the course of carcinogenesis, several substances related to the iron metabolism such as non-hemin iron, copper, catalase, ascorbic acid and riboflavin were investigated in the liver of rats fed p-dimethylaminoazobenzene (DAB).

The results obtained on the liver of rats fed DAB for 30 days were already reported in the previous paper of this series (1).

Further informations on the iron metabolism of rats fed DAB for 90-150 days will be given in this communication.

Material and Methods

Catalase activity of the liver homogenate was assayed and expressed in k as described in the previous report (1). Non-hemin iron of the liver was determined by the method of Yonekawa and Konno (2). Copper content of liver was estimated by the wet ashing method of Eden and Green (3). Total ascorbic acid was determined by the method of Roe *et al.* (4). Total flavin was determined by the fluorophotometric method of Yagi (5).

Rats were fed on a diet containing DAB (0.6 g of DAB in 20 ml of olive oil was mixed with one kg of rice) for about 90 and 150 days (6). Then the rats were killed by exsanguination and the determinations were carried out in the liver removed.

In the rats fed on DAB for 150 days there appear hepatomas. In these cases the hepatoma parts and the residue (cirrhotic part) were separated, and the determinations were carried out on each part. The "hepatoma part" looks white and rather hard, while the "cirrhotic part" appeared reddish, although it may contain hepatoma cells microscopically.

Results

I. *Liver Catalase Activities of Normal Rats and Those Fed DAB.* Fig. 1 shows liver catalase activities of normal rats and those fed DAB. The liver of rats fed DAB for 90 days has a lower catalase activity than the normal.

In the liver of rats fed DAB for 150 days, hepatomas were observed here and there, and the non-cancerous part of the liver was cirrhotic. The catalase activities of

hepatoma were always lowest, but that of non-tumorous part showed a somewhat different aspect.

Lower catalase activity was observed in the non-tumorous part of liver bearing large tumor, while those bearing small hepatoma showed rather higher catalase activity than the normal.

And this was also true in the liver of rats fed DAB for 150 days in which no hepatoma appeared.

II. *Iron Contents in the Liver of Normal Rats and Those Fed DAB.* Table 1 shows the non-hemin iron contents of the normal rat liver. In this table PI, PII, PIII and SIII represent hemosiderin, ferritin, nucleoprotein bound, and free iron, respectively.

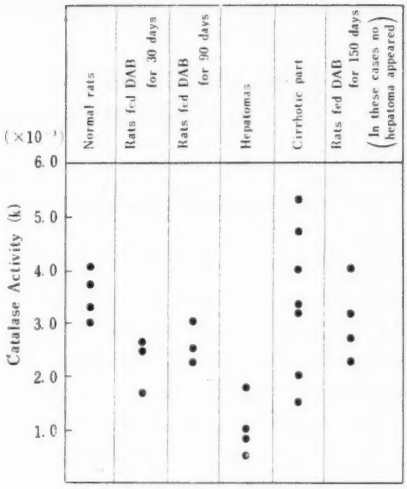

Fig. 1. Catalase Activity in the Liver of Normal and DAB Fed Rats.

Table 1. The Iron Content in the Normal Rat Liver
(expressed by γ/g of wet liver)

Exp. No.	PI	PII	PIII	SIII
1	31	72	16	—
2	52	140	10	—
3	34	66	4	—
4	41	70	16	—
5	34	66	13	—
6	32	65	16	—
7	26	115	5	—

Table 2. The Iron Content in the DAB Fed Rat Liver for 90 days
(expressed by γ/g of wet liver)

Exp. N.	PI	PII	PIII	SIII	Catalase Activity
25	91	70	22	9	2.3×10^{-3}
26	98	110	27	7	2.5×10^{-3}
28	132	46	43	13	3.0×10^{-3}

Table 3. The Iron Content in the Liver of the Rat Fed DAB for 150 days
(expressed by γ/g of wet liver)

Exp. No.	Hepatoma Parts				
	PI	PII	PIII	SIII	Catalase Activity
55	31	11	32	11	0.5×10^{-3}
45	49	28	66	13	0.8×10^{-3}
51	107	42	36	15	1.5×10^{-3}
58	122	50	96	5	1.0×10^{-3}

Table 4. The Iron Content in the Liver of Rat Fed DAB for 150 days
(expressed by γ/g of of wet liver)

Exp. No.	Non-tumorus Parts				
	PI	PII	PIII	SIII	Catalase Activity
55	54	18	69	9	1.8×10^{-3}
45	135	41	95	20	1.5×10^{-3}
58	122	250	19	10	3.3×10^{-3}
51	290	138	36	15	4.7×10^{-3}
60	70	130	20	13	3.2×10^{-3}
62	73	122	20	15	4.0×10^{-3}

Tables 2, 3 and 4 show the non-hemin iron contents of the liver of rat fed DAB for 90-150 days.

As shown in these tables, hemosiderin iron (PI fration) keeps higher value than normal during DAB-carcinogenesis. After 90 days' feeding, nucleoprotein bound iron (PIII fraction) increased gradually and the hepatoma tissue had the highest value.

The ferritin contents (PII fraction) of the hepatoma was definitely lower than the normal, but in the non-tumorous part of the liver bearing hepatoma there is a somewhat curious situation. Those bearing large tumor have a lower value of PII fraction than the normal, but those bearing small hepatoma have a higher value than the normal. And as shown in Fig. 2, there was some parallelism between ferritin contents and catalase activities in these non-tumorous part of the liver bearing large hepatoma.

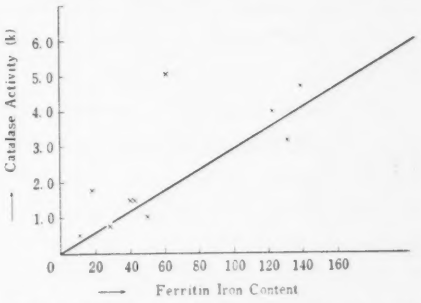

Fig. 2 Parallelism between catalase Activity and Ferritin Iron in the Liver of Rats Fed DAB for 150 days.

III. *Total Copper Content in the Liver of Normal Rats and Those Fed DAB.* Table 5 shows the copper content of the liver of the normal and DAB fed rats (90 days). As shown in the table copper contents of the liver of rats fed DAB for 90 days show a lower value than the normal.

IV. *Total Ascorbic Acid Contents in the Liver of Normal Rats and Those Fed DAB.* As shown in Tables 6 and 7, the total ascorbic acid contents of the liver are always kept higher than the normal during the carcinogenesis, and this is also the case even in the induced hepatoma.

V. *Total Riboflavin Contents of Normal Rats and Those Fed DAB.* Table 8 shows the total riboflavin content in the liver of normal rats and those fed DAB for

Table 5. Liver Copper Contents of Normal and DAB Fed Rats (90 days)
(expressed by γ/g of wet liver)

Normal rat		DAB fed rat		
Exp. No.	Copper content	Exp. No.	Copper content	Catalase activity
1	19.5	7	4.2	1.8×10^{-3}
3	25.0	8	6.2	2.6×10^{-3}
5	17.4	9	8.4	2.7×10^{-3}
6	26.5	11	5.6	3.3×10^{-3}

Table 6. Total Ascorbic Acid Content in the Liver of Normal and DAB Fed Rats (90 days)
(expressed by γ/g of wet liver)

Normal Rat		DAB Fed Rat		
Exp. No.	Total Ascorbic Acid	Exp. No.	Total Ascorbic Acid	Catalase Activity
9	230	36	340	1.8×10^{-3}
11	174	37	350	2.6×10^{-3}
12	224	38	340	2.7×10^{-3}
14	248	39	330	3.3×10^{-3}

Table 7. Total Ascorbic Acid Content in the Liver of DAB Fed Rats (150 days)
(expressed by γ/g of wet liver)

Exp. No.	Part of Liver	Total Ascorbic Acid	Catalase Activity
45	hepatoma	315	0.8×10^{-3}
72	〃	283	1.4×10^{-3}
75	〃	315	0.5×10^{-3}
45	non-tumorous	307	1.8×10^{-3}
72	〃	300	5.3×10^{-3}
75	〃	300	2.0×10^{-3}

90 days. As shown in the table, total riboflavin contents in the liver of rats fed DAB for 90 days are on the same level as the normal, while that of rats fed DAB for 30 days was clearly lower than the normal (1). As shown in Table 9, total riboflavin in the hepatoma is always lower than the normal, while that in the non-tumorous part is higher than the normal.

Table 8. Total Flavin Content in the Liver of Normal and DAB Fed Rats (90 days)
(expressed by γ/g of wet liver)

Normal Rat		DAB Fed Rat		
Exp. No.	Total Flavin	Exp. No.	Total Flavin	Catalase Activity (k)
1	13.5	36	10.1	1.8×10^{-3}
2	11.6	37	14.0	2.6×10^{-3}
3	12.1	38	12.5	2.7×10^{-3}
		39	9.0	3.3×10^{-3}

Table 9. Total Flavin Content in the Liver of Rats Fed DAB for 150 days
(expressed by γ/g of wet liver)

Exp. No.	Part of Liver	Total Flavin	Catalase Activity
45	hepatoma	8.3	0.8×10^{-3}
72	〃	4.8	1.4×10^{-3}
45	non-tumorous	17.6	1.8×10^{-3}
75	〃	14.1	5.1×10^{-3}
51	〃	9.7	4.7×10^{-3}

Discussion

Present communication together with the previous paper (1) has now given a complete aspect on the changes of several substances related to the iron metabolism in the course of carcinogenesis by DAB.

Hemosiderin iron contents (PI) in the non-tumorous part of the liver maintained the level above the normal during carcinogenesis, but that of induced hepatoma was in the normal range.

The changes of ferritin iron contents (PII) and the catalase activities in the non-tumorous part of the liver during carcinogenesis are quite puzzling. The ferritin contents increased at first and then decreased, but again increased after the development of small cancer nodules. The catalase activity dropped at first and then approached normal, but it became above normal after the appearance of small hepatoma.

Although these strange phenomena are unexplainable at present, the initial drop of catalase activity can not be attributed to the toxohormone function anyway.

The definitely lower values of catalase and ferritin iron in both hepatoma and the non-tumorous part of liver bearing large hepatoma may be attributed to the toxo-

hormone produced by the cancer tissue. Because, the toxohermone prepared from cancer tissues, when injected into mouse, caused parallel depression of catalase activity and ferritin iron in the liver (7).

The contents of nucleoprotein bound iron (PIII) increased gradually after 3 months' feeding, and reached maximum in the hepatoma and the non-tumorous part of liver bearing hepatoma. This fact may indicate that the increase of nucleoprotein is an indispensable process for the induction of cancer cell.

A gradual decrease of the copper content and the high ascorbic acid content in both non-tumorous part of liver and hepatoma are also unexplainable phenomena at present.

Up to one month, the DAB feeding leads to the decrease of total riboflavin of liver, but after that this vitamin gradually increases. This phenomenon has an inverse relation to the polar dye formation which is in maximum in thirty days' DAB feeding.

In this connection, the following fact is worth noticing that DAB feeding leads to an increased excretion of riboflavin in the animal urine, but non-carcinogenic aminoazobenzene (AB) has a far weaker effect in elimination of riboflavin than DAB (8). This fact may indicate some intimate relation between flavoprotein and DAB metabolism.

Summary

1) Rats were fed on DAB diet for 90–150 days, and catalase, nonhemin iron, copper, ascorbic acid, and riboflavin were determinted in the rat liver.

2) Hemosiderin iron in the non-cancerous part of liver maintained the level above normal during carcinogenesis, but that in the induced hepatoma was in the normal range.

3) Nucleoprotein-bound iron increased gradually after 3 months' feeding and reached maximum in the hepatoma and non-tumorous part of liver bearing hepatoma.

4) Ferritin iron of liver during carcinogenesis increased at first and then decreased, but again increased after the appearence of small cancer nodules. When large hepatoma appeared, ferritin iron in the non-tumorous part of liver was lowered under the normal. The ferritin iron content of hepatoma had the lowest value.

5) Catalase activity in the cirrhotic part of liver dropped at first, and then approached normal, but it became above normal after the appearance of small cancer nodules. Both hepatoma and non-tumorous part of liver bearing large hepatoma had definitely lower catalase activities than the normal.

6) The copper content of liver decreases gradually during DAB carcinogenesis.

7) Ascorbic acid in hepatoma and in the non-cancerous part of liver is maintained the level above normal during carcinogenesis.

8) Riboflavin in the hepatoma induced by DAB was less than that in the normal

liver. During carcinogenesis, however, the lowest riboflavin content was observed in the liver of rat fed DAB for 30 days, and then the content of this vitamin approached normal.

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REFERENCES

1) Doi, G., Gann, **48,** 243 (1957).
2) Yonekawa, M., and Konno, K., J. Biochem., **40,** 377 (1940).
3) Eden, A., and Green, H.H., Biochem. J., **34,** 1202 (1940).
4) Roe, T.H., and Oesterling, M. J., J. Biol. Chem., **152,** 511 (1944).
5) Yagi, K., Vitamins, **6,** 528 (1953).
6) Miyaji, T., Ozaki, K., and Nakazima, I., Igaku to Seibutugaku, **11,** 4 (1947).
7) Hoshizima, H., Gann, **48,** 239 (1957).
8) Okuda, K., Unpublished data.

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STUDIES ON THE MECHANISM OF LIVER-CARCINOGENESIS BY CERTAIN AMINOAZO DYES. II. THE HEPATIC FORMALDEHYDE DEHYDROGENASE AND XANTHINE OXIDASE ACTIVITIES OF RATS AND MICE IN RELATION TO THE DAB CARCINOGENESIS

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INTRODUCTION

The carcinogenic potency of dimethylaminoazobenzene (DAB) is influenced by many factors. Addition of riboflavin, liver powder[2], blood powder[3], high content of protein[4] or some hydrocarbons[5] in the diet have been reported to reduce the carcinogenicity of DAB remarkably. The mechanism of the inhibitory effect of these substances is not clear enough, but may partly be ascribed to the altered metabolic activity of the experimental animals against the carcinogens. It has been reported[6][7] that the metabolism of DAB and other aminoazo dye carcinogens is deeply related with the carcinogenic mechanism of these dyes.

The three main types of the metabolism referred to the carcinogenic aminoazo dyes are reductive cleavage at the azo group, hydroxylation at the aromatic rings, and oxidative demethylation at the amino group.

If we stand upon the hypothesis[8] that the dye-protein binding *in vivo*, followed by the deletion of the corresponding proteins, is at least one of the mutational mechanisms of the normal cells into the tumor cells, any metabolism leading to the dye-protein binding should be the important carcinogenic metabolism. Other metabolisms which are not related to the binding mechanism might be called detoxicating metabolisms conventionally.

According to the results mostly obtained by the Millers et al.[9], the presence of N-methyl group is considered to have an important role in the carcinogenesis by the aminoazo dyes. Terayama et al.[10] proposed the possibility that the formaldehyde (in statu nascendi), the primary product in the oxidation of N-methyl group, is responsible for the binding between the dye and the tyrosine group of the protein. The active formaldehyde concentration in the course of the carcinogenic aminoazo dye metabolism could be very important therefore. The formaldehyde generated *in vivo*, however, can be metabolized further. The metabolic pathway through tetrahydrofolic acid to the labile methyl pool seems to be not so important[11][12] in this case, because the incorporation of C^{14} labeled N-methyl of DAB into serine or choline *in vivo* was

rather small and most of the N-methyl metabolized was shown to be expelled in the form of CO_2. The dehydrogenating pathways of formaldehyde to CO_2 through the formic acid and formaldehyde dehydrogenases seems to be the main metabolic pathway concerned herewith. If the hepatic formaldehyde thus formed by the oxidative demethylase[13)14)] can be promptly oxidized further and the possibility for the active formaldehyde to bind the dye with the certain proteins becomes less. The role of the hepatic xanthine oxidase in relation to the carcinogenesis[15)] is a rather complicated problem. The liver xanthine oxidase may oxidize formaldehyde[16)]. The possibility for the xanthine oxidase to reduce the azo group[9)17)] has been assumed and the anticarcinogenic effect of dietary riboflavin was considered to be due to the strengthened xanthine oxidase (a flavoprotein) activity. This possibility, however, seems to be very doubtful[18)19)20a)20b)]. Recently the xanthine oxidase was closed up with respect to the iron utilization *in vivo*, namely, the reduction of ferritin iron coupled with the xanthine dehydrogenase activity[21)22a)]. The administration of DAB has been reported to reduce the hepatic riboflavin[22b)] and catalase[23)], while the hepatic hemosiderin and ferritin increased[24)]. It is also very interesting to see that the hepatic xanthine oxidase itself is one of the metalloflavo proteins which are reduced in the activity by the administration of DAB[19)25)]. The uric excretion of riboflavin was also reported[33)].

In this report the enzymic activities of formaldehyde dehydrogenase and xanthine oxidase of the liver of normal rats, mice and a guinea pig were compared. The effect of the administration of some aminoazo dyes with a help of a stomach tube upon the enzymic activities were also compared.

The results obtained thus far indicated that rats which are most susceptible to the DAB carcinogenesis showed the low activity in both formaldehyde dehydrogenase and xanthine oxidase in the liver, compared with mice which are more resistant to the DAB carcinogenesis. The administration of DAB caused a great decrease in the hepatic xanthine oxidase and formaldehyde dehydrogenase activities but the effect of MAB was less and the administration of AB did not show any decreasing effect. The inhibitory effect of DAB administration was observed more significantly with rat liver than with mouse liver.

Experimental

1. Formaldehyde Dehydrogenase

Animals. Wistar albino rats (100–120g, male) and albino mice (15–18g, male) were used. The administration of the dye dissolved in olive oil or propylene glycol was carried out with a help of a stomach tube under anesthesia with ether. The animals were then fed the standard diet* (product of the Oriental Yeast Co., Ltd.) or rice as indicated in each case of the experiments for three days before the sacrifice. As

* Protein 250g, carbohydrate 520g, fat 60g, salt mixture 100g and water 70g.

the control the animals were administered with olive oil or propylene glycol without dyes and the enzymic activities were compared between the test and the control.

Preparation of the Enzyme. Immediately after the decapitation of the animals the liver was perfused *in situ* with ice cooled 0.9 per cent NaCl solution. 1-1.5g of the liver was homogenized at 0°C for two or three minutes in a Potter-Elvehjem homogenizer after mixed with nine times as much of the 0.9 per cent NaCl added with a few drops of toluene. The homogenate thus prepared was dialysed against M/5000 versene for 24-29 hrs. In the absence of versene the aldehyde dehydrogenase activity was greatly reduced.

Assay of the Enzyme Activity. The Thunberg tubes were used for the assay. The composition of the reaction mixture was as follows:

Main chamber	M/10 Pyrophosphate buffer (pH 9.6)	1 ml
	Enzyme (Liver homogenate, dialysed)	1 ml
	Coenzyme I (0.2 per cent) solution	0.4 ml
	Nicotinamide (1M)	0.2 ml
	Water	0.4 ml
Side chamber	Formaldehyde (0.02 per cent)	1 ml
	Triphenyltetrazolium chloride (TTC) (0.001M)	1 ml
	Total	5 ml

Three tubes were prepared for the assay of the test enzymes. Two tubes for the control which were devoid of the formaldehyde were also prepared. In the latter cases one ml of water was added instead of the formaldehyde solution. To make it sure that the formation of the pink TPF (triphenylformazan) formed during the incubation is an enzymic process, one tube was prepared using the heated liver homogenate. No pink color was formed in this case. The reaction was carried out in a constant temperature bath regulated at 37°C for two hours. At the end of the reaction, 0.5ml of trichloroacetic acid (TCA) was added to stop the reaction. 7ml of ethylacetate was added to extract the formed formazan (TPF). The supernatant ethylacetate layer after the centrifugation was

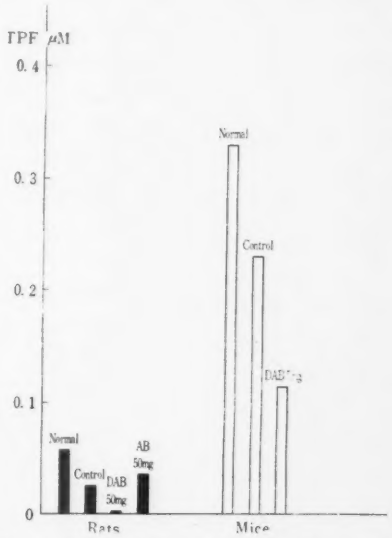

Fig. 1 Effect of Dye Administration upon Hepatic Formaldehyde Dehydrogenase Activity.

pipetted and the optical density at 485mμ was measured using a Beckmann spectrophotometer.

In order to relate the optical density with the amount of TPF formed, a standard curve was prepared in advance, using the various amounts of TTC, reduced with sodium hydrosulfite and then extracted with 7ml of ethylacetate. The dry weight of 10 per cent homogenate was measured and the amount of the formazan produced per 20mg dry weight was compared as shown in the Figure 1.

The Effect of Dye Administration upon the Formaldehyde Dehydrogenase Activity. As shown in Table 1, the dehydrogenase activity of mice is higher than that of rats (about ten times as much). The activity is partly reduced (by about 50 per cent) by the control treatment (olive oil or propylene glycol administration without dye), but the administration of DAB made the activity further reduced (almost down to ten per cent of the control), especially with rats liver. It is very interesting that the administration of AB does not reduce the formaldehyde dehydrogenase activity of both rats and mice liver.

The formaldehyde dehydrogenase activity of guinea pig liver was almost neglisible

Table 1. Comparison of Formaldehyde Dehydrogenase Activity of Liver Homogenate

Treatment		Relative Activity (TPF μ moles/20mg dry liver)	
Animals: Rats			
No treatment (standard diet)		0.066	
Propylene glycol (rice)	2ml	0.019	
Olive oil (rice)	1ml	0.028 0.022 0.035 0.021	Av. 0.026
DAB 50mg in olive oil (DAB 0.06% rice)	1ml	0.000 0.003	Av. 0.002
MAB 50mg in olive oil (DAB 0.06% rice)	2ml	0.004	
MAB 25mg in olive oil (standard diet)	1ml	0.030	
AB 50mg in olive oil (rice)	1ml	0.035	
Animals: Mice			
No treatment (standard diet)		0.330	
Propylene glycol (rice)	0.2ml	0.083	
Olive oil (rice)	0.2ml	0.382	
Olive oil (rice)	0.1ml	0.126 0.221 0.360 0.209	Av. 0.229
DAB 5mg in olive oil (DAB 0.06% rice)	0.1ml	0.179 0.049	Av. 0.114
DAB 3.3mg in propylene glycol (DAB 0.06% rice)	0.2ml	0.000	

Guinea pig liver did not show any appreciable formaldehyde dehydrogenase activity. The food in the brackets in the table was fed for three days before the sacrifice of the animal.

even when the animal was fed the standard diet long before the assay.

The formaldehyde dehydrogenase activity was reduced when the animals were fed only rice instead of the standard diet which contains adequate amounts of nutritional requirements[26].

The formaldehyde dehydrogenase activity was almost completely inhibited by disulfiram[27] ($6.8\times10M^{-4}$, I=100 per cent) and by KCN ($3.3\times10M^{-4}$, I=85 per cent) under the experimental conditions reported here.

2. Xanthine Oxidase

Animals. The care and treatment of the experimental animals were same with the above description for the experiments of formaldehyde dehydrogenase.

Preparation of the Enzyme. Three days after the administration of the dye with a help of a stomach tube the animals were killed by decapitation. The liver perfused immediately in situ with ice cooled 0.9 per cent NaCl solution. 4-5g of the fresh liver was homogenized with two times as much of water added with two or three drops of toluene in a Potter-Elvehjem homogenizer (4 minutes at 0°C). The homogenate was dialysed against running water for four hours and then against one liter of distilled water at 0°C for 20 hours.

Assay of Xanthine Oxidase Activity. The decoloration time measurement of methylene blue in the Thunberg tube was used for the assay of the xanthine oxidase activity. The reaction mixture had the following composition:

Main chamber:	0.5M Phosphate buffer (pH 7.5)	0.2 ml
	Enzyme solution	2.0 ml
Side chamber:	0.5 per cent Hypoxanthine in dil. KOH	0.2 ml
	0.0113M Methylene blue	0.1 ml

The general technique for the assay was carried out according to Richert *et al.*[28] Two tubes were prepared for the assay and two tubes were also prepared without hypoxanthine as the control. The reaction was started after two minutes of temperature equilibrium period in the constant temperature bath regulated at 37°C. The time needed for the decoloration of methylene blue was measured by seconds. In order to relate the decoloration time with the conventional xanthine oxidase activity, the data which indicate the relation between the decoloration time and QO_2 by Westerfeld *et al.*[28] were used. Two ml of the enzyme solution were evaporated and the dry residue was weighed.

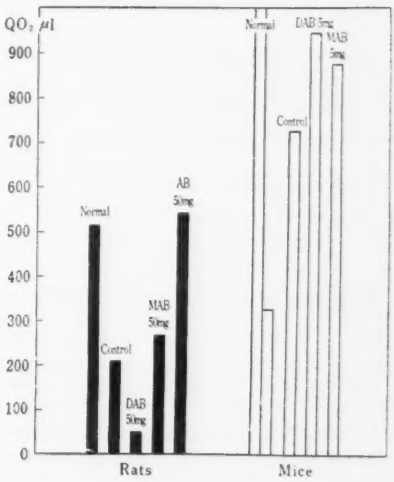

Fig. 2. Effect of Dye Administration upon Hepatic X.O. activity.

Effect of Dye Administration on the Xanthine Oxidase Activity. DAB, MAB and AB dissolved in olive oil or propylene glycol were administered intragastrically with a help of the stomach tube. The liver of the experimental animals were removed three days later, homogenized and the xanthine oxidase activity was compared with the control, to which only the solvent oil without dye was administered. The results are shown in Table 2 and Figure 2.

As shown in Table 2, the xanthine oxidase activity of mouse liver is higher than that of rat liver (about one and half times as high). The xanthine oxidase activity

Table 2. Comparison of Xanthine Oxidase Activity of Liver

Treatment	MB decoloration time (Hypoxanthine) with (min : sec)	MB decoloration time (Hypoxanthine) without (min : sec)	X.O. Activity (QO_2/100mg dry liver)	
Animals: Rats				
No treatment (standard diet)	4 : 15	29 : 40		
	4 : 30	39 : 00		
	5 : 00	—		
	4 : 30	16 : 00	534	Av. 512
	5 : 13	11 : 20	364	
	4 : 00	10 : 30	638	
Propylene glycol 2ml (rice)	11 : 00	45 : 00	227	
Olive oil 1ml (rice)	7 : 30	24 : 30	367	Av. 206
	11 : 30	46 : 00	—	
	12 : 45	34 : 30	141	
	13 : 50	44 : 25	111	
DAB 50mg in olive oil 1ml (DAB 0.06% rice)	17 : 00	26 : 00	48	
DAB 33.4mg in propylene glycol 2ml (DAB 0.06% rice)	15 : 00	47 : 00	81	
MAB 50mg in olive oil 2ml (DAB 0.06% rice)	8 : 05	36 : 00	336	Av. 268
	8 : 30	14 : 30	200	
MAB 25mg in olive oil 1ml (standard diet)	4 : 55	14 : 47	538	
AB 50mg in olive oil 1ml (rice)	6 : 00	30 : 00	541	
Animals: Mice				
No treatment (standard diet)	3 : 25	15 : 00	1490	Av. 1325
	3 : 00	31 : 50	1160	
Propylene glycol 0.2ml (rice)	4 : 30	42 : 30	709	
Olive oil 0.1ml (rice)	5 : 33	39 : 10	523	Av. 721
	3 : 20	42 : 30	1120	
	4 : 50	44 : 00	837	
	7 : 23	51 : 30	403	
DAB 5mg in olive oil 0.1ml (DAB 0.06% rice)	5 : 00	41 : 45	648	Av. 943
	3 : 20	38 : 40	1330	
	3 : 50	46 : 00	1210	
	5 : 28	39 : 30	585	
DAB 3.3mg in propylene glycol 0.2ml (DAB 0.06% rice)	4 : 20	42 : 15	734	
MAB 5mg in olive oil 0.2ml (DAB 0.06% rice)	3 : 43	20 : 45	873	
MAB 5mg in olive oil 0.2ml (standard diet)	5 : 00	28 : 15	570	
Animals: Guinea pigs				
No treatment (standard diet)	9 : 25	66 : 30	250	

of both rat and mouse livers decreased as the result of the administration of propylene glycol or olive oil (one half). It has been reported that the xanthine oxidase activity was also shown to decrease in the case of low protein diet[29)30)]. The administration of a great amount of DAB with a help of a stomach tube significantly decreased the xanthine oxidase activity of the rat liver (about one quarter of the control), but the administration of MAB was almost of no effect and the AB administration rather increased the activity with rat liver. The mouse liver was rather insensitive towards the azodye administration. The xanthine oxidase activity of a guinea pig liver was comparatively weak.

Xanthine Oxidase Activity of Various Tissues of Rats. The various rats fed the standard diet were used for the comparison of the xanthine oxidase activity of tissues. The preparation and the assay technique were the same as the above. The result is shown in the following table (Table 3).

Table 3. Xanthine Oxidase Activity of Rats Tissues

Tissue	MB decoloration time (Hypoxanthine)		X.O. Activity (expressed in QO_2 μ1)	Dry weight of Enzyme used (mg)
	with (min : sec)	without (min : sec)		
Liver	4 : 30	16 : 00	770	146
Spleen	10 : 10	43 : 50	260	85
Kidney	15 : 20	17 : 50	50	101
Testis	34 : 00	100 : —	0	70

Inhibition of Xanthine Oxidase The following substances were examined as the inhibitors against the rat's xanthine oxidase. The result is shown in Table 4.

Table 4. Inhibition of Rats Hepatic Xanthine Oxidase

Inhibitors	Final conc. (M)	MB decoloration time (min : sec)	Degree of inhibition (per cent)
Acrinamin	0.0001	10 : 38	43
Folic acid	0.0001	32 : 38	97
KF	0.01	9 : 38	10
PCMB	0.0074	no decoloration	100
Control I	No inhibitor	8 : 50	
Control II	No inhibitor No hypoxanthine	19 : 00	

It is clear that folic acid and PCMB are very strong inhibitors towards the hepatic xanthine oxidase. The inhibition by the folic acid used for this study may probably be due to the contamination of the decomposition product, namely, 2-amino-4-hydroxy-6-pteridyl aldehyde[31)].

Effect of the Folic Acid upon the Metabolism of MAB by Rats Liver. The rat liver homogenate fortified with TPN and DPNH etc. was used. The general technique

of the assay of the metabolic products as well as the experimental conditions were described in the preceding paper[7].

The result is shown in the following table (Table 5).

Table 5. Analysis of the Metabolic Products of MAB by Liver Homogenate

	MAB (remained)	AB (formed)	OH-Dye*(formed)	Dye decomposed**
Control	2.2 (γ)	21.0 (γ)	4.1 (γ)	72.7 (γ)
Folic acid (0.00016M)	1.2	21.3	4.6	72.9

Incubated at 37°C for 30 minutes. DPNH showed the higher activity than DPN. The initial amount of MAB incubated is 100γ.

*Hydroxylated dyes were calculated in terms of 4'-hydroxyaminoazobenzene.

**This value indicates roughly a measure of the azo group reduction.

As shown in Table 5, the folic acid used here showed no inhibition toward the azo group reduction, while the same material showed the strong inhibition against the xanthine oxidase at the lower concentration. This fact appears to show that the hepatic xanthine oxidase is not involved in the azoreduction.

Discussion

It is our hypothesis that the formaldehyde which is the first step metabolic product of N-methyl oxidation might be responsible for the binding between the dye and some special proteins in the liver fed the dye. The demethylase activity therefore could be one of the most interesting metabolic processes with respect to the carcinogenesis. It has been shown by Conney et al.,[13] and also confirmed by us, that the demethylase activity of mouse liver is higher than that of rat liver when 3-Me-MAB was used as a substrate.

Suppose the metabolic path from N-methyl group to formaldehyde is a carcinogenic metabolism, then there would be many other anticarcinogenic (or detoxicating) metabolisms at the same time. The azo reductase which breaks up the azo dye molecules at the site of azo group is one of them. Aldehyde dehydrogenase which oxidizes formaldehyde to formic acid could be another. It is known that aldehyde can be oxidised either by xanthine oxidase (a metalloflavoprotein) or by the specific DPN dependent aldehyde dehydrogenase. Another metabolic path which reduces the formaldehyde concentration in liver is via tetrahydrofolic acid depending labile C_1-transferring system. As far as the metabolism of butter yellow and its related aminoazo dyes are concerned, this last metabolic path does not seem so important.

Hydroxylation of aromatic ring[32] and subsequent glucuronide or sulfuric acid ester formation could also be important detoxicating factors which counteract the effect of the carcinogenic metabolism.

If the above hypothesis is right, any organ or animal which is susceptible to the

butter yellow carcinogesis should have the ability to oxidize N-methyl group and this has been proved experimentally. Then how can it be explained that the mouse liver having the higher N-demethylase activity than rat liver is less susceptible to the butter pellow carcinogenesis than rat liver? The answer for this question seems to be at least partly presented by our study reported here. The anticarcinogenic metabolisms in general are much higher with mouse liver than with rat liver. The very strong xanthine oxidase and aldehyde dehydrogenase activities may reduce the effective formaldehyde concentration which may act both as cytotonic and as mutagenic agent.

The remarkable decrease in xanthine oxidase and formaldehyde dehydrogenase observed three days after the admininistration of DAB is very interesting if we consider that the results obtained by many people[22b)23)] showing the decrease of riboflavin in the liver by the administration of DAB. Such decrease in the enzymatic activities was not observed when AB was administered. This fact may suggest that the N-methyl metabolism is responsible for the decrease in the enzymatic activities. The fact that the mouse liver which is resistant to the carcinogenesis is more tolerant against the effect of the dye administration may be understood by considering the high detoxicating activity of the mouse liver. The fact that the folic acid sample used in this study inhibited completely the xanthine oxidase activity but the same sample did not show any inhibition upon MAB metabolism even at the higher concentration may be interpreted as showing that the xanthine oxidase is different from the azo reductase.

The decreased activity in the xanthine oxidase of rat liver as the result of DAB administration may block the utilization of ferritin and this may be in turn reflected in the decrease of the iron containing enzymes and the increase of ferritin and hemosiderin contents in the liver[33)].

Summary

1) The DPN dependent formaldehyde dehydrogenase activity (FDH) and xanthine oxidase (X.O.) activity of rat and mouse liver were compared. The mouse liver showed several times as high enzymic activities as those of rat liver. This observation was considered to explain at least partly why mouse liver forms much less protein bound dye and is less sensitive to the dye carcinogenesis compared with rat liver.

2) With rat liver, the intragastric administration of the carcinogenic DAB greatly diminished FDH activity but the non-carcinogenic AB had no decreasing effect. The FDH activity of mouse liver was also decreased by the administration of DAB, but to less degree compared with rat liver.

3) X.O. activity of rat liver was decreased by the administration of DAB. MAB showed almost no effect and AB appeared to increase the X.O. activity of both rat and mouse liver. X.O. activity of mouse liver was not altered by the administration

of DAB or MAB.

4) The metabolism of N-methyl group of aminoazo dye seems to be responsible for the drastic decrease in FDH and X.O. activities in the rat liver.

5) The evidence was presented to show that the azo group reductase is different from the xanthine oxidase.

6) The meaning of N-methyl metabolism with respect to the protein binding of the carcinogenic aminoazo dyes was discussed.

References

1) C.J. Kensler, K. Sugiura, N.F. Young, C.R. Halters and C.P. Rhoads: Science **93,** 208 (1941)
2) W. Nakahara, K. Mori and T. Fujiwara: Gann **33,** 241 (1939)
3) W. Nakahara and F. Fukuoka: Gann **38,** 428 (1944)
4) J.A. Miller, D.L. Miner, H.P. Rush and C.A. Baumann: Cancer Res. **1** 699 (1941)
5) E.C. Miller, J.A. Miller, R.R. Brown and J.C. MacDonald: Cancer Res. **18,** 469 (1958)
6) E.C. Miller and J.A. Miller: Cancer Res. **6,** 39 (1946)
7) H. Terayama: Gann **49,** 223 (1958)
8) E.C. Miller and J.A. Miller: Cancer Res. **7,** 468 (1947)
9) J.A. Miller and E.C. Miller: Advances in Cancer Res. **1,** 353 (1953)
10) H. Terayama, K. Kusama, K. Teruya, S. Kuroda and T. Nakayama: Gann **49,** 85 (1958)
11) E.C. Miller, J.C. MacDonald and J.A. Miller: Cancer Res. **15,** 320 (1955)
12) R.A. Boissonnas, R.A. Turner and V. du Vigneaud: J. Biol. Chem. **180,** 1053 (1949)
13) A.H. Conney, R.R. Brown, J.A. Miller and E.C. Miller: Cancer Res. **17,** 628 (1957)
14) J.R. Gillet, B.B. Brodie and B.N. LaDu: J. Pharmacol. Exptl. Therap. **119,** 532 (1956)
15) I. Lewin, R. Lewin and R.C. Bray: Nature **180,** 763 (1957)
16) E.C. DeRenzo: "Advances in Enzymology" Vol. 17, 293 (1956)
17) W.C.J. Ross and G.P. Warwick: Nature **176,** 298 (1955)
18) J.R. Fouts, J.J. Kamm and B.B. Brodie: J. Pharmacol. and Exptl. Therap. **120,** 291 (1957)
19) G.C. Mueller and J.A. Miller: J. Biol. Chem. **180,** 1125 (1949)
20a) "Ciba Foundation Symposium on the Chem. and Biology of Purine." p. 259-265, (1957),
20b) K. Deij: Acta Physiol Pharmacol. Neerl. **7,** 106 (1958)
20c) A.C. Griffin and C.A. Baumann; Arch. Bioch. **11,** 467 (1946)
J. & A. Churchill Ltd. London.
21) M.M. Weber, H.M. Lenhoff and N.O. Kaplan: J. Biol. Chem., **220,** 93 (1956)
22) S. Green and A. Mazur: J. Biol. Chem. **227,** 653 (1957)
23) I. Yanagizawa: Gann **36,** 234 (1942)
24) G. Doi: Gann **48,** 243 (1957)
25) J.M. Price and G.M. Weber: Cancer Res., **9,** 398 (1949), **10,** 18, 28 (1950)
26) D.A. Richert and W.W. Westerfeld: J. Biol. Chem., **227,** 533 (1957)
27) M.L. Keplinger and J.A. Wells: J. Pharmacol. Exptl. Therap. **119,** 19 (1957)
28) D.A. Richert and W.W. Westerfeld: J. Biol. Chem. **181,** 266 (1949)
29) W.W. Westerfeld, D,A. Richert, and M.F. Hilfinger: Cancer Res. **10,** 486 (1950)
30) J.P. Greenstein and A. Meister: Sumner and Myrbaeck, "The Enzymes, Chem. & Mechanism of Action." Vol. 11 p. 1158 Academic Press.
31) H.M. Kalckar, N.O. Kjeldgaard and H. Klenow: J. Biol. Chem. **174,** 771 (1948)
32) J. Booth and E. Boyland: Biochem. J. **66,** 73 (1957)
33) S. Mizuhara: Proceedings of the General Meeting of the Japanese Cancer Soc., November (1958)

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UPTAKE AND CONSERVATION OF RADIOACTIVE ZINC BY EHRLICH ASCITES CARCINOMA CELLS

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INTRODUCTION

Although the occurrence of natural zinc has been established in human and other mammalian tissues (1-9), little is known concerning the function of zinc in cell-physiology, except that zinc has been shown to be an essential component in several enzyme systems (10-14). A possible role in mitosis has been suggested by Fujii (15) on the basis of histochemical informations that zinc is present in nucleoli and is transfered into chromosome and spindle during the mitosis time.

On the other hand, zinc content in some tumour tissues has been found to be higher or lower than that of normal tissue (16-22). It has been reported by Tupper *et al.* (23) that the tumour tissue (mammary gland carcinoma) had a small but statistically significant higher ^{65}Zn content than had normal mammary gland tissue of the same mouse. In both the spindle-cell sarcoma and mammary adeno-carcinoma of mice studied by Heath *et al.* (24), the malignant tissue took up more of the ^{65}Zn than the normal tissue supporting tumour growth.

In the present paper, it is shown that Ehrlich ascites carcinoma cells take up ^{65}Zn at very much higher concentration than do some tissues of tumour-bearing and normal mice and that the ^{65}Zn in tumour cells is maintained for a fairly long period of time.

EXPERIMENTAL METHODS

Tumour: The tumour used was Ehrlich ascites carcinoma transplanted in the inbred ddN strain of male and female mice, weighing about 20 g. Transplantations were always done by intraperitoneal inoculation with about 0.1 ml of the ascites fluids of a seven day carcinoma.

Preparation of Zinc Solution: ^{65}Zn was supplied in solution as chloride with an excess of hydrochloric acid from the Oak Ridge National Laboratoy. The zinc solution for injection was prepared by the method of Tupper *et al.* (23) as follows: after dissolving 20 mg of glycine in 0.44 N hydrochloric acid solution of $^{65}Zn\,Cl_2$ (3 mc), it was adjusted to pH 7.4 (Phenol Red) by adding 1 N sodium hydroxide and rendered

isotonic by addition of sodium chloride and then was made up to a final volume of 100 ml with distilled water to form the stock solution. The final stock solution contained 3.99 μg of zinc/ml and had 30 μc of radioactivity/ml.

Injection of Zinc Solution: Each mouse received 0.3 ml of the ^{65}Zn containing zinc-glycine complex solution on the fifth day after the transplantation of tumour. The injections were made subcutaneously below the dorsal surface or intraperitoneally. The normal non-tumour bearing mice received in the same manner the injection of ^{65}Zn solution for control experiments.

Preparation of Tissue Samples: On the first, the third and the seventh days after the injection of zinc solution, the mice were killed with blows or by cervical dislocations and immediately the ascites fluids and other required tissues were removed by blunt-dissection. The ascites fluids were centrifuged. The supernatant (ascites plasma) and the recovered cells washed with hypertonic sucrose solution were pooled for the determinations of radioactivity and nitrogen.

Labeling of Tumour Cells by ^{65}Zn: The injections of ^{65}Zn-containing zinc-glycine complex solution were made intraperitoneally once on the fifth day and twice on the sixth day after the transplantation of tumour cells. On the next day of the last injection of ^{65}Zn, the ascites fluids were harvested by repeated rinsing of the abdominal cavity of the host mouse with small volume of Krebs-Henseleit solution containing citrate. The ascites fluids combined with the washings of cavity were centrifuged for 5 min. at 3000 r.p.m. and the recovered cells were resuspended in warm Krebs-Henseleit solution. This suspension of ^{65}Zn-labeled tumour cells was used for the transplantations.

Determination of Radioactivity: The sample was digested with 5 ml of conc. sulfuric acid added with a small amount of both potassium and copper sulfates in the micro-Kjeldahl flask and then the radioactivity of the digest was measured for 3 min. with a scintillation counter (I.D.L. Co., England) using thallium activated sodium iodide as a scintillater. When the injection of zinc solution was done, the radioactivity standard was prepared by taking 1 ml from the solution for injection.

Determination of Total Nitrogen: Total nitrogen was determined by the micro-Kjeldahl method.

Experimental Results

Subcutaneous Injection of ^{65}Zn: Fig. 1 shows the radioactivities recovered in Ehrlich ascites tumour cells and liver of the mice which received subcutaneous injection of ^{65}Zn below the dorsal surface. The ratio of the radioactivity of tumour cells to that of liver is shown in Fig. 2. On the first day, the uptake of ^{65}Zn by ascites tumour cells is lower than that by livers of the tumour-bearing and normal mice, and then it gradually increases and after one week reaches a higher value

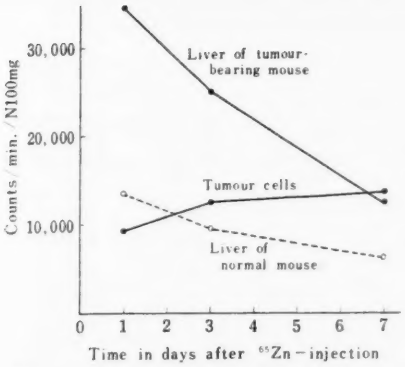

Fig. 1. The radioactivities recovered in Ehrlich ascites carcinoma cells and livers of the tumour-bearing and normal mice, each of which received 9 μc of the ^{65}Zn-containing zinc-glycine complex solution subcutaneously below the dorsal surface.

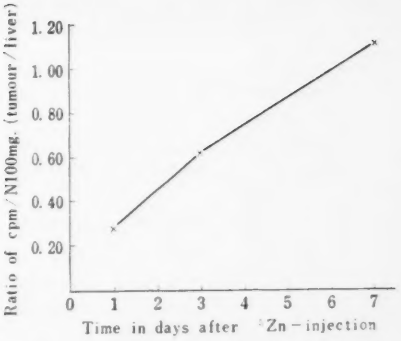

Fig. 2. The ratio of radioactivity recovered in Ehrlich ascites carcinoma cells to that in liver in the tumour-bearing mouse, which was calculated by using the data presented in Fig. 1.

than that of liver.

Intraperitoneal Injection of ^{65}Zn: The results where the ^{65}Zn-containing zinc-glycine complex solution was injected intraperitoneally are shown in Tables 1 and 2 and Fig. 3. The ^{65}Zn was rapidly incorporated into the tumour cells and its activity was an average of 38000 counts/min./100 mg of nitrogen on the first day. It is above six-fold as large as the average counts recovered in the livers of tumour-bearing and normal mice, but rapidly decreases with the lapse of days. This result is contrast to that of subcutaneous injection. As shown in Table 1, however, the radioactivity recovered in the ascites plasma was no more than one-tenth of that in the tumour cells in both cases of the subcutaneous and intraperitoneal injections.

Labeling of Tumour Cells by ^{65}Zn and the Fate of Labeled Tumour Cells in

Table 1. The radioactivities recovered in Ehrlich ascites carcinoma cells and ascites plasma after the intraperitoneal injection of ^{65}Zn.

Days after injection of ^{65}Zn	Ehrlich ascites carcinoma cells (c.p..m/100mg N)	ascites plasma (c.p.m./100mg N)
1	38012 (4)	3387 (4)
3	23501 (4)	1966 (4)
7	8803 (4)	641 (4)
10	8968 (3)	771 (3)

Each mouse received 9μc of the ^{65}Zn-containing zinc-glycine complex solution on the fifth day after the inoculation of tumour. Figures given are averages of analyses from 3 to 4 mice. The number of mice examined is shown in parentheses.

Table 2. The radioactivities recovered in the various tissues of normal and tumour-bearing mice after the intrapenitoneal injection of the ^{65}Zn solution.

Days after injection of ^{65}Zn	Liver		Kidney		Spleen	
	Normal mouse	Tumour-bearing mouse	Normal mouse	Tumour-bearing mouse	Normal mouse	Tumour-bearing mouse
1	5798 (3)	6073 (4)	4874 (3)	3947 (4)	6409 (3)	3689 (4)
3	3251 (3)	4261 (4)	2305 (3)	2479 (4)	2854 (3)	2109 (4)
7	3105 (3)	3898 (4)	1202 (3)	2099 (4)	1475 (3)	2282 (4)
10	1279 (2)	3784 (3)	770 (2)	2532 (3)	767 (2)	2267 (3)

Each mouse received 9μc of the ^{65}Zn-containing zinc-glycine complex solution on the fifth day after the inoculation of Ehrlich ascites carcinoma. The radioactivity was expressed as c.p.m./100mg nitrogen The figures are averages of analyses from 2 to 4 mice. The number of mice examined is shown in parentheses.

the Body of Mouse: On the basis of the fact that the incorporation of ^{65}Zn reaches already the maximum value on the first day after the injection of zinc solution, the method of labeling of tumour cells by ^{65}Zn was adopted according to that described under "Experimental Methods". The suspension of ^{65}Zn-labeled cells in Krebs-Henseleit solution used for the intraperitoneal inoculation had the activity of 4570 counts/min./ml. Table 3 and Fig. 4 show the radioactivity recovered in the ascites tumour cells after the intraperitoneal inoculation of 0.3 ml of the ^{65}Zn-labeled cells suspension per mouse. The ^{65}Zn in the tumour cells is very rapidly lost during

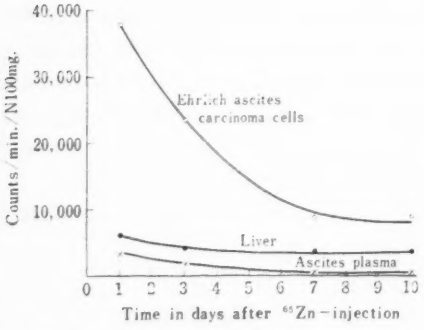

Fig. 3. The radioactivities recovered in Ehrlich ascites carcinoma cells and its ascites plasma and in the liver of the tumour-bearing mouse after the intraperitoneal injection of the ^{65}Zn-containing zinc-glycine complex solution on the fifth day after the inoculation of tumour.

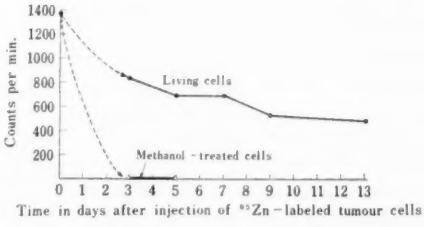

Fig. 4. The fate of ^{65}Zn injected intraperitoneally with the form of ^{65}Zn-labeled Ehrlich ascites carcinoma cells and the effect of methanol treatment of the cells before the injection.

Table 3. The radioactivities recovered in the tumuor cells and liver of the tumour-bearing mouse after the intraperitoneal inoculation of the ^{65}Zn-labeled Ehrlich ascites carcinoma cells.

Days after inoculation of labeled cells	Ehrlich ascites carcinoma cells (c.p.m./whole population of cells)	Liver of tumour-bearing mouse (c.p.m./whole tissue)
3	842 (3)	42 (3)
5	696 (3)	56 (3)
7	681 (3)	38 (3)
9	534 (3)	75 (3)
13	482 (4)	63 (4)

The figures given are averages of analyses from 3 to 4 mice. The number of mice examined is shown in parentheses.

the first two days and then the remaining ^{65}Zn is gradually lost on the latter days. As shown in Fig. 8, the graph of radioactivity in logarithmic scale *versus* time in days becomes a straight line during the period of gradual loss, and its tangent is negative but very small, namely, -0.0562 c.p.m. (log units) per day. The half-value period of this gradual loss is estimated to be 12.3 days. This suggests that the ^{65}Zn in the tumour cells is conserved during a fairly long period following the initial loss. When mice were injected intraperitoneally with the suspension of ^{65}Zn-labeled tumour cells which were treated by methanol, the washings of abdominal cavity showed already no activity after three days, though the activity recovered in the liver was nearly equal to that of the mice bearing the ^{65}Zn-labeled carcinoma.

Discussion

Calculation of Excretion Coefficient : According to Nieset (25), the concentration of a tracer isotope in any given metabolic compartment can be represented by a polynomial function with exponential terms. So let us assume that the specific radioactivity (S) recovered in a tissue at time t is given by the equation:

$$S=S_1e^{-\alpha t}+S_2e^{-\beta t}, \tag{1}$$

where S_1, S_2, α and β are characteristic constants of the tissue. At time zero ($t=0$) when an animal receives the isotope, the tissue away from the site of injection contains no radioactivity, that is, $S_1+S_2=0$. Putting $S_1=-S_2\equiv S_0$, Eqn. (1) is rewritten as

$$S=S_0(e^{-\alpha t}-e^{-\beta t}), \tag{2}$$

where S_0 characterizes the behaviours of the tissue. In the case of $\alpha<\beta$, the maximum activity will be found at time t_m which is expressed as

$$t_m=\frac{1}{\beta-\alpha}\ln(\beta/\alpha) \tag{3}$$

It may be considered that the coefficients α and β indicate the apparent abilities of excretion and uptake of the isotope by the tissue, respectively. If $\alpha \ll \beta$, the radioactivity will reach the maximum value in the short period after the injection of isotope and then will decay closely approximating to the equation:

$$S = S_0 e^{-\alpha t} \quad \text{or} \quad \ln S = \ln S_0 - \alpha t \tag{4}$$

so that the graph of $\ln S$ *versus* t becomes a straight line (Fig. 5). The half-value period τ of this decay curve is given by

$$\tau = \frac{1}{\alpha} \ln 2 = 0.69315/\alpha \tag{5}$$

If the time course of radioactivity recovered in any given tissue approximates to Eqn. (4) after the activity has reached the maximum, the apparent ability of excretion of the isotope by the tissues can be compared with each other by using the constant α or τ.

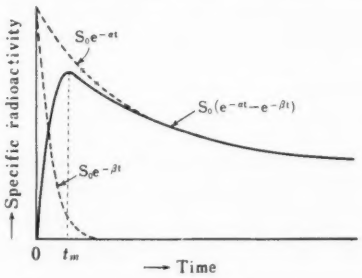

Fig. 5. A curve of the time course of the specific activity recovered in a tissue away from the site of injection of the isotope, which approximates closely to Eqn. (2).

In both the normal and tumour-bearing mice, the radioactivities recovered in the liver, kidney and spleen after the intraperitoneal injection of the ^{65}Zn-containing solution reach the maximum value on the first day and then decrease approximating to Eqn. (4) (Fig. 6). The apparent excretion coefficient α and half-value period τ of each tissue are calculated by using the data presented in Table 2 and summarized in Table 4. As shown in Table 4, the apparent excretion of ^{65}Zn by the tissues is delayed by bearing the ascites carcinoma, and the delay effect is the smallest in liver and the largest in spleen among the tissues examined. What has caused this delay effect? An answer for this question can be given by the following consideration.

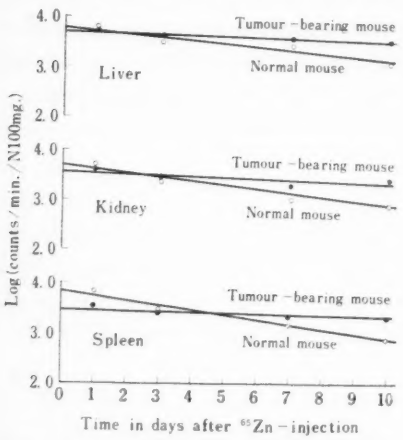

Fig. 6. Apparent excretion of the ^{65}Zn by the various tissues of Ehrlich ascites carcinoma-bearing and normal mice. The data are given in Table 2.

In the present paper, the radioactivity is expressed as counts per min. per 100mg of nitrogen of tissue. Hence,

$$S = \text{c.p.m./100 mg of nitrogen} = \frac{\text{total c.p.m./whole tissue}}{\text{total nitrogen/whole tissue}} = \frac{Z^*}{N} \tag{6}$$

where Z^* and N are the total amounts of ^{65}Zn and nitrogen contained in the whole

Table 4. The apparent excretion coefficient and half-value period of various tissues of Ehrlich ascites carcinoma-bearing and normal mice for the ^{65}Zn injected intraperitoneally.

		Apparent excretion coefficient (c.p.m. (log units)/day)	Biological half-value period (days)	Delay effect*
Liver	Normal mouse	0.1455	4.8	3.1
	Tumour-bearing mouse	0.0470	14.7	
Kidney	Normal mouse	0.1943	3.6	4.5
	Tumour-bearing mouse	0.0431	16.1	
Spleen	Normal mouse	0.2211	3.1	5.9
	Tumour-bearing mouse	0.0380	18.2	

* The delay effect is defined as the ratio of half-value period of the tissue of tumour-bearing mouse to that of normal one.

tissue, respectively. From Eqn. (4), the apparent excretion coefficient is rewritten as

$$\alpha = -\frac{d}{dt}\ln\left(\frac{Z^*}{N}\right) = \frac{1}{N}\cdot\frac{dN}{dt} - \frac{1}{Z^*}\cdot\frac{dZ^*}{dt} \tag{7}$$

Putting

$$\frac{1}{N}\cdot\frac{dN}{dt} = \mu \quad \text{and} \quad \frac{1}{Z^*}\cdot\frac{dZ^*}{dt} = -\bar{\alpha}, \tag{8}$$

the apparent excretion is expressed as the sum of these coefficients, that is

$$\alpha = \bar{\alpha} + \mu, \tag{9}$$

where $\bar{\alpha}$ is the actual excretion coefficient. This difference between the apparent and actual excretion coefficients can be clearly seen in the ascites tumour cells. Using the data summarized in Tables 1 and 3, both apparent and actual excretion coefficients of Ehrlich ascites carcinoma cells are calculated to be 0.2599 c.m.p. (log units) per day between the sixth and 12th days and 0.0562 c.p.m. (log units) per day between the

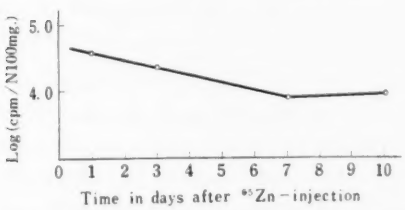

Fig. 7. Apparent excretion of ^{65}Zn from Ehrlich ascites carcinoma cells. Each mouse received the ^{65}Zn by intraperitoneal injection on the fifth day after tumour inoculation. The data used are given in Table 1.

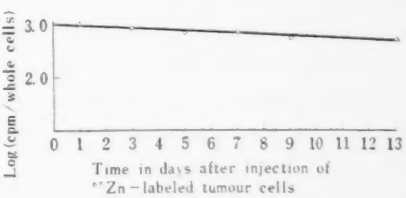

Fig. 8. Actual excretion of ^{65}Zn from Ehrlich ascites carcinoma cells. Each mouse received the ^{65}Zn with intraperitoneal inoculation of the ^{65}Zn-labeled tumour cells. The data used are given in Table 3.

third and 13th days after the inoculations of tumour, respectively (Figs. 7 and 8). The value of μ is estimated to be 0.2037 mg (log units) per day between the sixth and 12th days after the tumour inoculation. Assuming that the nitrogen content per cell is constant throghout the life of the cell and in any part of the tissue, the μ is equal to the specific rate of multiplication of cells being represented as $(1/C)\,dC/dt$, where C is the total number of cells. It is therefore concluded that the rate of multiplication per day to the total number of Ehrlich ascites carcinoma cells may be 20.4% between the sixth and 12th days after the inoculation. Since the actual excretion coefficient is practically constant between the third and 13th days, the value of apparent excretion coefficient α may be larger than 0.2599 c.p.m. (log units) per day between the third and fifth days, that is, at an early stage of proliferation of the tumour cells where the specific rate of multiplication must be larger than the value obtained above.

In a normal adult animal, the tissue may be in a stationary state, namely $\mu=0$, then the apparent excretion coefficient is equal to the actual one itself. During the course of our experiments, the hypertrophy has been observed in the spleen of tumour-bearing mice. This is also seen in the leucemia-bearing mice. In the tumour-bearing mice, the tissues may be under conditions of non-stationary state where the nitrogen content or the number of cells composing the tissue is increasing or decreasing. Assuming the μ is zero during the observation, however, the apparent excretion coefficient is also equal to the actual one itself in the tumour-bearing mice. From these points should be examined the delay effect of excretion observed in the tumour-bearing mice. In any case, it may be said that the delay effect is attributed to the gradual excretion of the isotope from the ascites tumour cells.

Comparison with the Fate of Tubercle Bacilli in the Body of Mouse: It is confirmed in the present experiments that the ^{65}Zn in the *living* ascites tumour cells intraperitoneally injected is conserved during a fairly long period, of which the half-value period is about 12.3 days, but the ^{65}Zn in the *dead* cells is very rapidly lost from the abdominal cavity of mouse (Fig. 4). This striking difference in the behaviour of ^{65}Zn will be available as a screening method of the anti-tumour reagents. The further studies concerning this possibilitiy will be pursued in our laboratory.

It is most interesting to refer the previous work of Yamamura *et al.* (26), one of the authors, that stands in sharp contrast to the result mentioned above. The behabiour of radioactive phosphorous in the mouse which received the ^{32}P-labeled tubercle bacilli by intravenous injection into the caudal vein was investigated and it was demonstrated that in the case of the virulent bacillus H37Rv strain, the ^{32}P in the body of mouse is gradually lost irrespective of *living* or *dead* of the bacilli injected. This result suggests that the cancer problem could not be treated from the viewpoint of analogy to the phenomena of infection or parasitism.

Summary

The uptake and conservation of ^{65}Zn by Ehrlich ascites carcinoma cells were studied by means of the subcutaneous and intraperitoneal injections of ^{65}Zn and the labeling of tumour cells by ^{65}Zn. The results obtained are as follows.

1. The ^{65}Zn injected subcutaneously below the dorsal surface of the tumour-bearing mouse was incorporated into the liver and the other tissues and then gradually transfered into the ascites tumour cells.

2. In the case of intraperitoneal injection, the ^{65}Zn was directly incorporated into the ascites tumour cells.

3. In both the subcutaneous and intraperitoneal injections, the ascites tumour cells took up more of the ^{65}Zn than the tissues of normal and tumour-bearing mice.

4. The ^{65}Zn incorporated into the tumour cells was conserved during a fairly long period, of which the biological half-value period was about 12.3 days.

5. The ^{65}Zn in the dead tumour cells was very rapidly excreted from the abdominal cavity of the mouse.

6. The ^{65}Zn-excretion from the tissues was delayed in the ascites tumour-bearing mouse.

References

1) Heath, J.C., B.E.C.C. Annual Reports, **16,** 198 (1939); **17,** 155 (1940); **19,** 46 (1942); **21,** 52 (1944); **25,** 127 (1947).
2) Rost, E., and Weitzel, A., Arb. Reichesundh Amt., **51,** 494 (1919).
3) Delezenne, C., Ann. Inst. Pasteur, **33,** 68 (1919).
4) Rost, E., Med. Klinik., **17,** 123 (1921).
5) Lutz, E., J. industr. Hyg., **8,** 177 (1926).
6) Dutoit, P., and Zbinden, C., C.R. Acad. Sci. Paris, **190,** 172 (1930).
7) Simakov, P.V., Bull. Biol. Med. exp. URSS, **10,** 258 (1940).
8) Eggleton, W.G.E., Biochem. J., **34,** 991 (1940).
9) Gettler, A., and Bastian, R., Am. J. Clin. Path., **17,** 244 (1947).
10) Keilin, D., and Mann, T., Nature, **144,** 442 (1939).
11) Vallee. B. L., and Neurath, H., J. Am. Chem. Soc., **76,** 5006 (1954).
12) Vallee, B. L., Adelstein, S.T., and Olson, J.A., *ibid.*, **77,** 5196 (1955).
13) Vallee, B.L., and Waker, W.E.C., *ibid.*, **78,** 1771 (1956).
14) Vallee, B.L., and Hoch, F.L., *ibid.*, **77,** 821 (1955).
15) Fujii, T., Nature, **174,** 1109 (1954).
16) Cristol, P., Bull. Soc. Chim. biol. Paris, **5,** 23 (1923).
17) Labbé, H., and Nepveaux, F., Progr. med., Paris, **54,** 577 (1927).
18) Sugai, M., Mitt. med. Akad. Kioto, **29,** 175 (1939).
19) Addink, N.W.H., Nature, **166,** 693 (1950).
20) Carruthers, C., and Suntzeff, V., J. Biol. Chem., **159,** 647 (1945).
21) Carruthers, C., and Suntzeff, V., Cancer Res., **6,** 296 (1946).

22) Cowdry, E.V., Carruthers, C., and Suntzeff, V., J. Nat. Cancer Inst., **8**, 209 (1948).
23) Tupper, R., Watts, R.W.E., and Wormall, A., Biochem. J., **59**, 264 (1955).
24) Heath, J.C., and Liquier-Milward, J., Biochim. Biophys. Acta, **5**, 404 (1950).
25) Nieset, R.T., Science, **122**, 742 (1955).
26) Yamamura, Y., Tani, J.. Terai, J., and Yamamura, Y. Amer. Rev. Tuber., **79**, in press.

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COPPER METABOLISM IN THE LIVER IN GASTRIC CANCER

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INTRODUCTION

Since it seems to be reasonable that the marked lowering of the synthesis of liver catalase cannot occur without concurrently involving changes in the general iron metabolism, many studies have been recently conducted concerning the catalase activity and iron metabolism in tumor-bearing animals. On the other hand, Adams[1] carried out extensive experiments on the maintenance of liver catalase activity in normal mice, and he arrived at the conclusion that the traces of copper and manganese are important in this mechanism. Not only iron and copper, but also some other trace elements, such as zinc and soforth, have begun attracting the attention of some students in cancer research field. We studied the metabolism of copper particulary in the liver in gastric cancer patients as well as in cancer-bearing animals.

EXPERIMENTAL METHOD

In surgical operation, we removed a piece of liver from patients as soon as the liver was explored, and measured its copper content, catalase activity, β-glucuronidase and ascorbic acid. Copper was estimated by carbamate method[2], catalase activity by gas volumemetry[3] using Battelli-Stern apparatus, β-glucuronidase by Fishman method[4] and ascorbic acid by both Roe[5] and indophenol methods. In measuring the copper content in the liver we used 20% trichloric acid according to Inoue and Takano[6] and separated the liver homogenates into two parts: the supernatant is called I-fraction, which is lightly bound copper fraction, and precipitate, II-fraction of strongly bound copper. We think that in physiological liver the I-fraction of copper is in good balance with the II-fraction. Accordingly, we paid much more attention to the relative changes of the I-fraction of copper.

RESULTS AND DISCUSSION

In gastric cancer both the catalase and β-glucuronidase activities of the liver seem to be lower than in gastric ulcer (Fig. 1 Table 1) while the I-fraction of copper and ascorbic acid higher (Figs. 2, 3, 4, 5 and 6, Tables 2, 3). We reported[7] already that in gastric cancer the liver catalase activity is in some inverse proportion to the I-

Table 1. Liver β-glucuronidase and catalase activities in gastritis, gastric ulcer and gastric cancer

Name	Sex	Age	β-glucuronidase activity mg/h. gm. w. w.	catalase activity O_2 c. c.
I. Gastritis & gastric ulcer				
1) K.N.	m.	22	30.5	16.7
2) I.O.	f.	65	26.9	10.5
3) E.A.	m.	50	16.6	22.8
4) K.I.	m.	34	17.4	10.0
5) S.A.	m.	61	18.3	18.2
6) E.K.	m.	66	24.8	15.6
7) M.I.	m.	56	17.3	23.3
Mean value			21.7	16.7
II. Gastric cancer				
1) T.K.	f.	58	12.4	15.5
2) K.N.	m.	65	20.5	19.5
3) M.O.	f.	50	13.3	10.2
4) M.H.	m.	61	11.9	5.2
5) F.K.	f.	42	15.1	12.4
6) M.K.	m.	59	18.2	12.3
7) K.K.	f.	45	17.9	11.6
8) Y.S.	m.	63	16.7	16.7
9) T.Y.	f.	29	15.2	10.4
Mean value			15.7	12.7

fraction of copper. It is interesting to mention our experiments which showed that also the ascorbic acid itself is in inverse proportion to the catalase activity, that is, the average value of ascorbic acid is 26.6 mg% when the catalase shows more than 10 cc activity. However, when the catalase activity shows less than 10cc, its average value of ascorbic acid increases to 33.6 mg%. When it comes to total ascorbic acid in the liver, we find that 4 cancer patients whose liver catalase activities are very low value have high value of ascorbic acid content. So both reduced and oxidised forms of ascorbic acid behave likeweise in our experiments (Table 3 a).

Ascorbic acid is oxidised by ascorbic acid oxidase, and the ascorbic acid oxidase is inhibited by copperic ion. Thus, we think that the results we obtained in gastric cancer show an intimate relation between I-fraction of copper and ascorbic acid. β-Glucuronidase is thought to be inhibited by copper, although it is not a SH enzyme. Our experimental results that liver β-glucuronidase is lower in gastric cancer than in gastritis or gastric ulcer point to the importance of I-fraction of copper in liver injury.

Toxohormone,[8] which was obtained by Nakahara and Fukuoka for the first time

Table 2. Liver ascorbic acid (reduced form), catalase activity and I-fraction of copper in gastritis, gastric or duodenal ulcer and gastric cancer

I. Gastritis, gastric or duodenal ulcer

	Name	Sex	Age	Ascorbic acid mg%	Catalase O_2 c. c.	I-fraction of Cu%
1)	K.S.	m.	51	10.2	8.1	76.1
2)	Y.H.	m.	27	10.9	13.7	73.3
3)	S.M.	m.	32	29.8	13.4	61.2
4)	S.K.	m.	39	30.6	16.8	65.6
5)	S.O.	m.	36	17.5	14.2	75.9
6)	T.T.	m.	40	23.3	9.8	62.0
7)	T.H.	m.	44	22.0	18.8	62.2
8)	K.T.	f.	25	29.6	15.6	61.7
9)	M.S.	f.	27	20.3	15.1	67.3
10)	N.H.	f.	27	22.8	18.7	67.4
11)	K.O.	f.	47	34.0	10.5	68.1
12)	C.M.	m.	28	24.4	9.2	79.7
13)	S.O.	m.	27	22.9	11.7	80.5
14)	Y.F.	m.	35	31.8	12.2	77.2
15)	T.M.	m.	51	37.1	11.5	75.0
16)	T.I.	m.	33	26.0	15.4	73.5
17)	S.E.	m.	49	20.1	14.2	69.3
18)	Y.N.	m.	69	23.7	11.3	66.1
19)	K.M.	m.	33	26.2	15.5	74.1
20)	N.G.	m.	53	27.2	12.2	Mean value 70.1
21)	Y.Y.	m.	38	25.1	23.4	
22)	M.I.	m.	56	27.4	11.5	
23)	N.S.	m.	56	28.2	13.6	
24)	S.A.	m.	21	23.1	14.1	Mean value of asorbic acid 1)—19) 24.4
25)	K.M.	m.	31	22.7	13.0	
26)	K.A.	m.	61	20.7	11.5	Mean value of catalase 1)—19) 13.5
27)	Z.S.	m.	52	28.1	11.5	
	Mean value			24.7	13.6	

II. Gastric cancer

1)	G.S.	m.	66	34.0	9.5	81.8
2)	C.S.	m.	66	31.7	11.5	78.6
3)	K.H.	m.	8.	15.0	15.0	77.1
4)	Z.Y.	m.	42	12.3	19.2	83.4
5)	Y.S.	m.	49	18.2	12.0	76.2
6)	K.S.	m.	52	23.5	12.4	69.0
7)	T.S.	f.	22	25.8	11.1	69.9
8)	K.M.	m.	59	37.0	17.9	62.3
9)	K.S.	m.	24	39.3	8.2	78.9
10)	S.W.	m.	55	30.9	9.0	81.0
11)	K.I.	f.	74	35.3	11.1	81.5
12)	T.I.	m.	45	32.6	5.4	80.0
13)	H.S.	m.	72	28.8	8.9	68.4
14)	K.Y.	m.	26	29.8	9.5	77.3
15)	T.Y.	f.	43	33.8	13.0	82.5
16)	M.I.	m.	38	32.2	16.2	78.8
17)	Y.A.	f.	72	30.7	7.9	78.9
18)	S.I.	m.	46	23.7	12.6	72.9
19)	K.N.	m.	54	31.6	27.1	62.3
20)	I.K.	m.	53	51.1	6.5	Mean value 75.8
21)	W.S.	f.	60	27.3	11.3	
22)	T.N.	m.	65	23.7	10.1	
23)	Y.K.	m.	62	23.8	6.6	Mean value of ascorbic acid 1)—19) 28.7
24)	K.K.	f.	55	25.4	13.9	
25)	T.F.	f.	54	25.5	14.3	Mean value of catalase 1)—19) 12.5
26)	I.H.	f.	58	24.8	7.7	
27)	T.H.	f.	65	45.0	5.5	
28)	T.M.	m.	54	26.4	11.1	
29)	T.S.	m.	66	34.8	10.6	
30)	S.M.	f.	60	32.9	8.0	
	Mean value			29.6	11.5	

Table 3a Liver ascorbic acid (total) and catalase activity in of gastritis, gastric ulcer and gastric cancer

Name	Sex	Age	Ascorbic acid mg %	Catalase activity O_2 c.c.
I. **Gastritis & gastric ulcer**				
1) N.G.	m.	53	20.0	12.2
2) YY.	m.	38	15.1	23.4
3) M.I.	m.	50	21.6	11.5
4) N.S.	m.	56	22.8	13.6
5) K.K.	m.	35	30.7	9.7
Mean valu			22.0	14.1
II. **Gastric cancer**				
1) W.S.	f.	60	28.6	11.3
2) T.N.	m.	65	22.1	10.1
3) K.K.	f.	55	17.0	13.9
4) T.I.	f.	22	33.0	7.0
5) K.K.	m.	55	21.0	11.6
6) E.T.	m.	60	27.5	8.2
7) K.N.	m.	63	20.5	16.3
8) A.I.	m.	64	24.2	8.1
9) S.K.	f.	61	22.0	6.4
10) Y.S.	m.	58	15.1	9.4
Mean value			23.1	10.2

Table. 3b Liver β-glucuronidase and catalase activities in toxohormone treated mice (Nakahara's preparation)

	β-Glucuronidase activity mg/h.·gm. w.w.	Catalase activity O_2 c.c.
Control		
1)	6.5	28.6
2)	6.4	28.5
3)	6.6	24.8
4)	7.0	19.7
Mean value	6.6	25.4
Toxohormone 15mg		
1)	4.8	11.9
2)	4.9	11.6
3)	4.4	18.3
4)	4.4	12.0
5)	4.7	9.1
Mean value	4.8	13.6
Toxohormone 20mg		
1)	4.2	13.3
2)	4.2	12.1
3)	3.6	10.3
4)	3.9	10.4
Mean value	4.0	11.5
Toxohormone 1)	3.6	16.1

must be a symbol of cancer cachexia. Although it is involved in normal tissue, we must attribute some part of cancer cachexia to it. Then, in order to clarify the action of toxohormone, we studied the relationship between toxohormone and copper,

Table. 4 Liver β-glucuronidase and catalase activity in Yoshida sarcoma hats

	β-Glucuronidase activity mg/h. gm. w.w.	Catalase activity O_2 c.c.
Control		
1)	21.4	46.2
2)	20.3	48.4
3)	22.7	30.6
4)	13.7	34.0
5)	22.1	36.0
6)	15.4	47.0
7)	26.0	53.8
8)	24.6	44.0
9)	21.4	49.0
10)	25.3	37.0
Mean value	21.3	42.6
Yoshida Sarcoma		
1)	13.5	15.7
2)	16.7	4.5
3)	18.0	9.7
4)	31.0	9.9
5)	23.0	13.7
Mean value	20.4	11.3

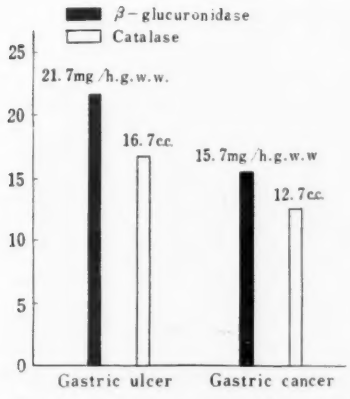

Fig. 1 Liver β-glucdronidase and catalase activity in gastric cancer and ulcer.

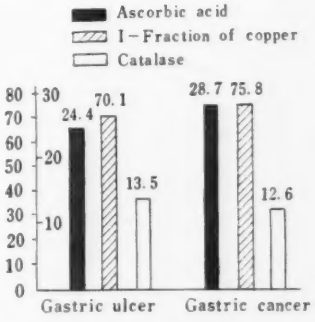

Fig. 2 Liver ascorbic acid (reduced form), catalase activity and I-fraction of copper in gastric cancer and ulcer.

ascorbic acid, β-glucuronidase, and catalase activity as described before. Toxohormone we used was provided for us by Dr. Nakahara and Prof. Masamune. Using many animals, we found the same tendency as above mentioned, that is in 24 hours after the intraperitoneal injection of both samples of toxohormone, the catalase activity and β-glucuronidase of liver decrease, (Figs. 7, 8 Table 3b) but the I-fraction of copper and ascorbic acid increase (Figs. 9, 10). But in tumor bearing animals, for instance, in Yoshida sarcoma rats (Fig. 11, Table 4) Ehrlich ascitic mice and in carbon tetrachloride injected mice (Figs. 12, 13, 14, Table 5) we cannot always obtain the same tendency as above described. For example, in Yoshida sarcoma rats and in carbon tetrachloride injected mice, no evident change was found in the liver β-glucuronidase during the period when liver catalase activity is of low value. In experimental chronically bled rats and castrated rats, however, the I-fraction of liver copper decreases and the catalase activity in liver is not changed.[7] In cortison

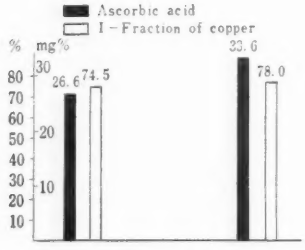

Fig. 3. Liver catalase activity, ascorbic acid (reduced form) and I-Fraction of copper in gastric cancer.

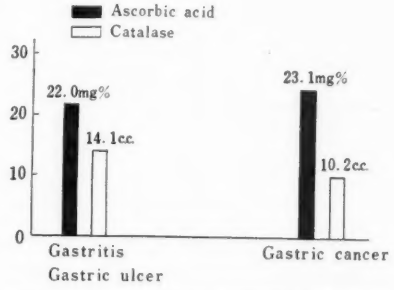

Fig. 4. Liver catalase activity and ascorbic acid (total) in gastric cancer, gastritis, gastric ulcer.

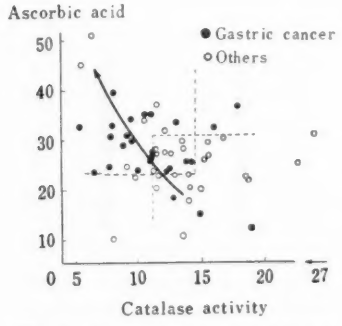

Fig. 5. Liver catalase activitiy and ascorbic acid (reduced form) in gastric cancer, gastritis and gastric ulcer.

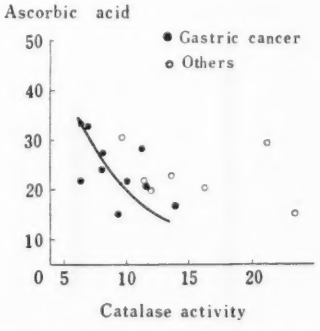

Fig. 6. Liver catalase activity and ascorbic acid (total) in gastric cancer, gastritis and gastric ulcer.

treated mice, which were given 0.25 mg daily for 15 days intraperitoneally, on the other hand, the liver catalase activity decreases, according to the increase of the I-fraction of copper in liver, but no change was found in ascorbic acid (Fig. 15). In brief, we suppose that the copper in the liver is very important not only in mechanism of action of toxohormone, but also in gastric cancer. In other words, it ocuppies the important part in cancer cachexia.

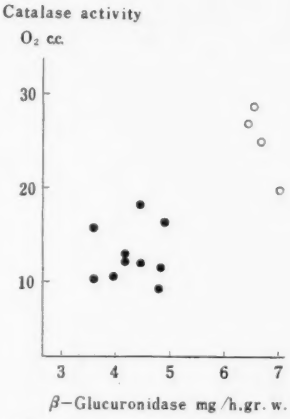

Fig. 7. Liver β-glucuronedase and catalase activity in toxohormone treated mice (Nakahara's preparation)

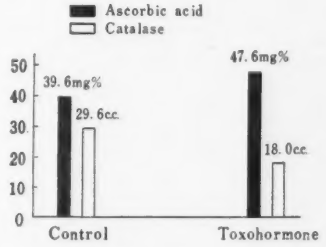

Fig. 8. Liver ascorbic acid and catalase activity in toxohormone treated mice (Nakahara's preparation 80 mg)

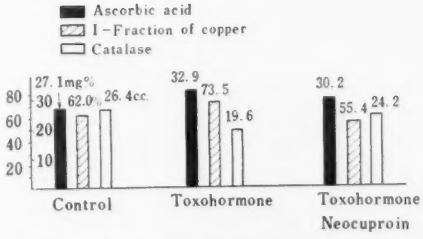

Fig. 9 Liver catalase activity, ascorbic acid (reduced form) and I-fraction of copper in Toxohormone and Neocuproin treated mice.

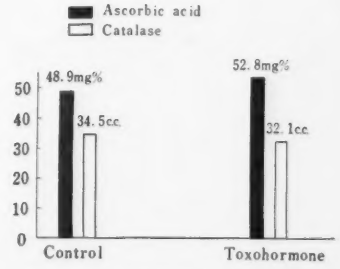

Fig. 10. Liver catalase activity and ascorbic acid (reduced form) in toxohormone treated mice (Masamune's preparation)

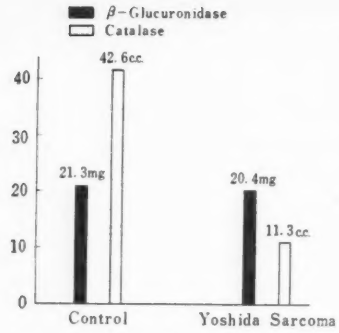

Fig. 11. Liver β-glucuronidase and catalase activities in Yoshida sarcoma rats.

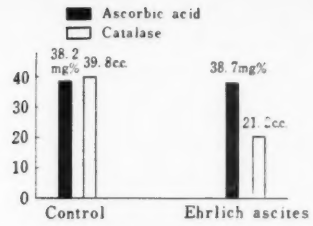

Fig. 12. Liver catalase activity and ascorbic acid (total) in Ehrlich ascitic mice on 10th day after implantation.

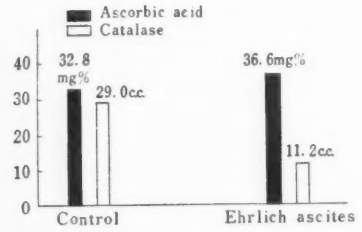

Fig. 13. Liver catalase activity and ascorbic acid (total) in Ehrlich ascitic mice on 15th day after implantation.

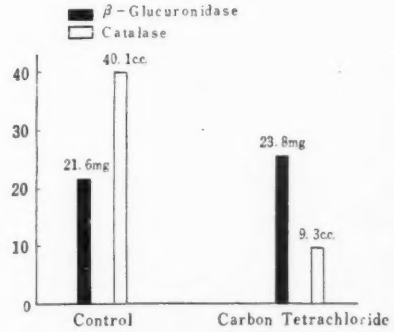

Fig. 14. Liver β-glucuronidase and catalase activity in carbon tetrachloride injected mice.

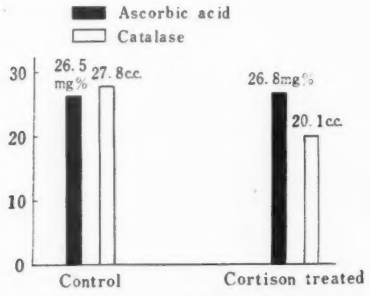

Fig. 15. Liver catalase activity and ascorbic acid in cortison treated mice (0.25 per a day for 15 days).

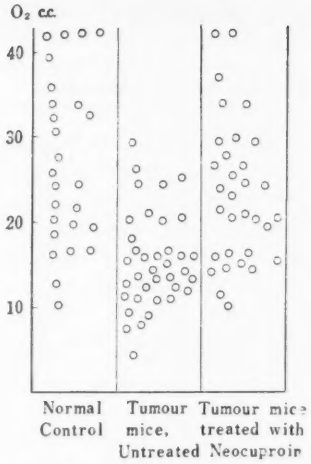

Fig. 16. Liver catalase activity in Ehrlich mice treated with Neocuproin.

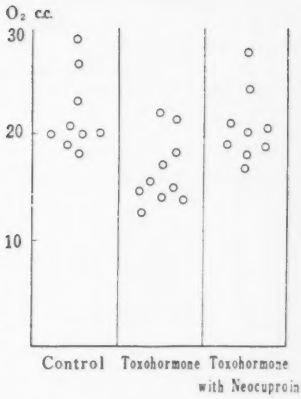

Fig. 17. Liver catalase activity in toxohormon treated mice with Neocuproin.

Then we thought that the copper chelating agents may give some influence upon the catalase activity in the liver. So we injected a specific chelating substance, Neocuproin (2, 9-dimethyl-1, 10-phenanthroline with 12.5 mg/kg W. in physiological saline solution[9]) into mice with Ehrilch ascitic carcinoma, finding that the lowering of liver catalase activity is to some degree prevented (Fig. 16). This substance was used 2 days after the tumor implantation. Moreover, this substance protects the mice from liver catalase activity lowering action of toxohormone (Fig. 17), without showing any anti-cancer action. At the same time, it also protects against the increase of ascorbic acid and I-fraction of copper in liver in toxohormone treated mice (Fig. 9). And it is more interesting that this substance has not any influence upon the liver catalase activity of normal mice (Fig. 18).

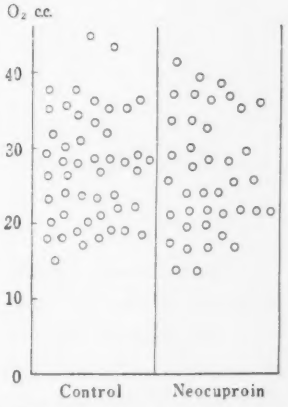

Fig. 18. Liver catalase activitiy in normal mice treated with Neocuproin.

We have already suggested that the decrease of liver catalase activity in tumor bearing animals may be owing not only to its disturbance of iron or porphyrin, but also of copper itself. Our present results speak more exactly for this fact.

Table. 5 Liver β-glucuronidase and catalase activities in carbon tetracloride injected mice.

	β-Glucuronidase activity mg/h.gm.w.w.	Catalase activity O_2 c.c.
Control		
1)	23.7	22.1
2)	22.7	34.9
3)	21.4	46.2
4)	20.3	48.4
5)	22.7	30.6
6)	13.7	34.0
7)	22.1	36.0
8)	15.4	47.0
9)	26.0	53.8
10)	24.6	44.0
11)	21.4	49.0
12)	25.3	37.0
Mean value	21.6	40.1
Carbon tetrachloride		
1)	25.2	10.0
2)	20.6	9.2
3)	26.1	7.6
4)	23.2	5.9
5)	24.9	16.7
6)	22.7	6.4
Mean value	23.8	9.3

SUMMARY

1) In the liver of gastric cancer, I-fraction of copper, that is labile copper fraction, increases in some inverse proportion to catalase activity.

2) The same results was not obtained in the liver in cancer bearing animals, such as Ehrlich ascitic mice and Yoshida sarcoma rats.

3) In the liver of gastric cancer patients and in toxohormone treated mice, β-glucuronidase decreases.

4) Neocuproin, which is thought to be a specific copper chelating substance, can inhibit the liver catalase activity lowering action of toxohormone, and the fall of liver catalase activity in tumor bearing animals may also be, to some grade, protected against.

5) This substance, Neocuproin, may be help to clarify the mode of action of toxohormone. Accordingly, copper may take very important role in the liver in gastric cancer. We think it may be of great significance in cancer cachexia.

The authors are indebted to Prof. S. Kimoto for his kind directions and also to Dr. W. Nakahara and Prof. Masamune for toxohormone preparations.

References

1) Adams, D.H.: Biochem. J. **50,** 486, 1952.
2) Cartwright, G.E.: J. Biol. Chem. **160,** 593, 1945.
3) Stern and Battelli: Oppenherimer and Pincussens, Die Methodik der Fermente **137,** 8, 1929.
4) Fishman, W.H.: J. Biol. Chem. **166,** 757, 1946.
5) Roe, J.H.: J. Biol. Chem. **147,** 379, 1943.
6) Inoue and Takano: Biochem. (in Japanese) **404,** 27, 1955.
7) Iijima, N.: Gann **47, 3,** 4, 267, 1956.
8) Nakahara W. and Fukuoka F.: Japan. Med. J. **1,** 271, 1948.
: Gann **40,** 45, 1949.
9) Smith, G, F.: Anal. Chem. **24,** 371, 1953.

[GANN, 50, 89~105; March, 1959]

STUDY OF SPLENIC ENLARGEMENT IN TUMOR-BEARING MICE

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INTRODUCTION

It has been pointed out by many investigators that the spleen shows a special response during the development of neoplasm. For example, the evidences that the spleen has little predilection for metastasis and that the splenectomized animal can be easily transplanted with tumor are recognized by many observers. Likewise, there are many reports concerning the splenic enlargement of animal bearing either primary or grafted tumors.

Roffo (1935)[1] found that the injection of blood of tumor-bearing rat could cause splenic enlargement in normal rats, while the blood of normal rat had no ability to cause such a change. Despite the fact that much information is available in this field of study, the reports are generally unrevealing. Therefore, it is considered worthwhile reconsidering the significance of splenic enlargement during tumor growth.

The author[2] while determining the DNA content of organs in mice bearing Ehrlich's carcinoma, found that the DNA (Pγ 100 mg wet tissue) in the spleen of tumor-bearing animals was lower in quantity when compared to that of control animals. These results may be of significance in explaining the splenic enlargement of tumor-bearing animals. The purposes of the present report are to ascertain whether splenic enlargement of the animals bearing Ehrlich's ascites carcinoma actually occurs and to elucidate the mechanism of the splenic response in such animals by using the estimation of their splenic DNA contents. In addition, the investigation was planned to determine whether the splenic enlargement occurred in normal mice after the injection of blood from tumor-bearing mice.

MATERIALS AND METHODS

1) *Experimental animal.* The animals employed in these experiments were healthy, male mice of d-d strain. Female mice were omitted because the splenic size of the female mouse is apt to be more frequently influenced by certain physiologic conditions such as pregnancy. Although it is desirable to use mice of same age, it was difficult to procure many such animals simultaneously. Also it is generally known that their sizes are more uniform in the younger age group.

However, small mice are inconvenient for various treatments due to minuteness of the organs, therefore, experiments were performed mainly on mice weighing somewhat more than 15 gm. All mice were sacrificed about six hours prior to feeding so as to prevent the splenic size from being influenced by the diet.

2) *Transplantable tumor.* The transplantable tumor, which was used in this experiment, was Ehrlich's ascites carcinoma. It was inconvenient to use this carcinoma in the ascitic form because the animals intraperitoneally transplanted with this tumor cannot live for more than an average of 14 days. Also the increase of ascites following the intraperitoneal implantation of the tumor cells may have other effects upon the spleen. Thus, Ehrlich ascites carcinoma was used as a subcutaneous tumor. All mice were killed 20 days after a definite number of free carcinoma cells was transplanted subcutaneously into the right gluteal region with a hypodermic syringe. After a specified period of tumor growth and immediately following decapitation of the animals, the tumor was extirpated as evenly as possible, and the weight of the residual body was recorded. Animals with externally necrotic tumor were discarded from the data.

3) *The measurement of splenic weight.* The spleen was extirpated usually by an abdominal incision and superflous blood around the organ was removed with a filter paper. The spleen was carefully weighed on a torsion balance. In the present study, actual weight (mg) of the organ was used and splenic index was not employed. The body weight of tumor-bearing animals was expressed as "Host weight−Tumor weight (gm)".

4) *The method employed in removing blood from the animal.* The blood was taken aseptically by cardiac puncture after an initial exposure of the thoracic cavities. The amount of blood obtained by this method was variable. Usually the volume withdrawn varied from 0.3 to 1.0 cc. The citrate solution was added to the blood to prevent coagulation. The supernate was used as the blood serum solution in the present study. Physiologic salt solution was added to the blood and later separated from the blood cells by centrifugation. The centrifugation procedure was repeated three times. Finally an adequate amount of physiologic salt solution was added to the blood cells and the number of red blood cells was counted in a counting chamber. The blood cells thus prepared were used for injections into hosts in this experiment. The blood was injected once only into the animals so as to avoid a bacterial contamination and the extirpation and recording of weight of the spleen was done on the 8th day following the inoculation of blood.

5) *The estimation of the DNA of the spleen.* The extraction of DNA from the organs were followed by a modification of Schneider's method. The procedure was carried out as follows[3]:

a) The splenic weight was determined immediately after removal from the animal, and the specimen was ground finely in a glass homogenizer with about 10 ml of absolute alcohol.

b) About 10 ml of acetone was added to the residue and the preparation was centrifuged two times.

c) To the residue about 10 ml of ether was added and reprecipitated two times. Ether was vaporized gradually from this material, and finally a completely dried powder of the organ was obtained. The weight of this powder corresponded to the defatted dry weight of the spleen. The estimation of DNA of the dry residue was done by Schneider's method (as modified by Shibano[4]), and the amount of phosphorous of the nucleic acid standard was estimated by the Fiske and Subbarow method[5]. The analytical values obtained from the specimens were expressed in γ of DNA-P per total splenic weight (mg) and per dry and wet weight (100mg) of the original spleen.

Results

1) *The change of splenic weight in tumor-bearing animals.* The correlation between the body weight and splenic weight of 118 normal mice is illustrated in Fig. 1. In expressing the unit of splenic volume, actual weight of the organ was used and not the splenic index. The usage of splenic index is undesirable in that it tends to increase the error in calculation. The correlation coefficient "r" obtained from the data in these animals is 0.47. When the reliability of the correlation coefficient is examined, the "t" value is 5.79, therefore, significiant correlation exists between body and spleen weights.

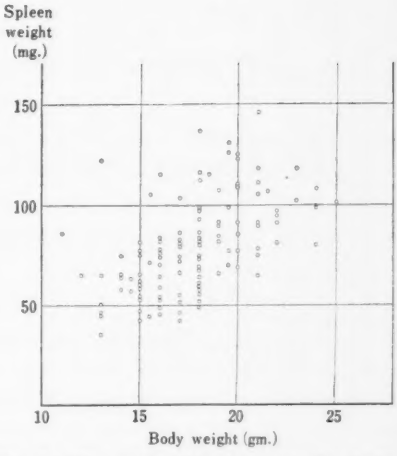

Fig. 1 The reationship between the body-weight and the spleen weight in 118 normal mice.

Next, in order to study the change of splenic weight, three groups of mice were inoculated subcutaneously with different amounts of free tumor cells. The mice of the first group, second group and third group received 5×10^6, 6×10^6 and 7×10^6 tumor cells respectively. Table 1 shows the relation between body weight and spleen weight of tumor-bearing animals in the three groups. As clearly shown by average values, the spleen weights show a more pronounced increase than in the control group.

Table 1. The measured values of body weight, spleen weight and tumor weight of animals inoculated subcutaneously with different numbers of tumor cells.

Mouse No.	Group 1. (inoculated with 5×10^6 tumor cells)				Group 2. (inoculated with 6×10^7 tumor cells)				Group 3. (inoculated with 7×10^6 tumor cells)			
	Host weight (gm)	(Host -tumor) weight (gm)	Tumor weight (gm)	Spleen weight (mg)	Host weight (gm)	(Host -tumor) weight (gm)	Tumor weight (gm)	Spleen weight (mg)	Host weight (gm)	(Host -tumor) weight (gm)	Tumor weight (gm)	Spleen weight (mg)
1	26	22	4	449	30	24	6	420	20	18	2	349
2	28	24	4	538	35	30	5	375	19	16.5	2.5	262
3	25	21.5	3.5	415	37	30	7	460	26	20.5	5.5	715
4	26	21.5	4.5	428	27	24	3	354	21.5	18	3.5	366
5	26	24	2	476	33	26	7	401	19.5	18	1.5	852
6	24	23	1	365	33	30	3	712	21.5	17	4.5	342
7	21.5	21	0.5	281	24	25.5	0.5	285	19	18	1	414
8	25	21	4	425	30	26	4	313	24	22	2	455
9	24	21	3	404	28	27	1	280	16	14	2	190
10	21	19	2	308	29	26.5	2.5	392	21	18	3	440
11	21	18	3	347	24	21.5	2.5	612	16	14	3	399
12	25	24	1	366	27	24	3	368	22	20	2	452
13	26	23	3	350	23	21	2	520	18	17	1	260
14	29	23	6	598	25	23	2	366	19	16	3	227
15	28	26	2	352	25	23	2	832	14	13.5	0.5	175
16	23	19	4	300	20.5	19	1.5	198	23	20	3	345
17	28	24	4	405	23	22	1	207	26	22	4	600
18	24	19	5	660	22	21	1	299	20	17	3	454
19	25	22	3	366	18	16	2	293	14	13	1	203
20	27.5	22	5.5	495	23	21	2	333	24	20	4	503
21	22	19	3	171	20.5	19	1.5	553	17	15	2	150
22	27.5	22	5.5	543	19	17	2	160	20	19	1	307
23	26	24	2	310	18	17	1	227	20	18	2	347
24	31	26	5	616	16	15	1	208	21	18	3	474
25	21	20	1	207	19	17	2	710	26	24	2	437
26	17	16	1	144	17.5	16	1.5	697	23	20	3	505
27	19.5	18	1.5	300	22.5	22.4	0.1	440	23	20	3	353
28	18	17.5	0.5	276	32	19	3	730	26.5	22	4.5	696
29	18	17	1	212	27	23	4	379	23	20	3	330
30	22	20	2	279	27	22	5	194	27.5	23	4.5	602
31	25	23.5	1.5	344	30	25	5	631	20	16.5	3.5	401
32	23	21	2	261	28	24	4	365	21	17	4	280
33	24	22	2	321	27	20	7	627	19	16	3	350
34	22	21	1	286	29	23	6	779	22	20	2	410
35	29.5	27	2.5	232	23	17	6	387	25	21.5	3.5	458
36	24	21	3	500	18	16	2	520	19	16	3	396
37	19.5	16	3.5	263	23	21	2	540	19	17.5	1.5	272
38	18	15.3	3.3	186	20.5	18.2	2.3	385	26	23	3	457
39	17.5	16.3	1.2	250	15	13	2	217	18.2	17.7	0.5	327
40	27.5	26.7	0.8	269	22	20.1	2.1	337	27	23.5	3.5	433
41	24	23.4	0.6	217	17.5	15.3	2.2	231	25.5	22.5	3	434
42	20	16.6	0.4	228	26	22.8	3.2	248	26	21.8	4.2	475
43	32	31	1	380	14.5	14	0.5	90	19	17.5	1.5	272
44					18	16.5	1.5	296	21	19.5	1.5	189
45					25	23.5	1.5	359	25	24.9	0.1	202
46					77	25.5	1.5	383	24	23.9	0.1	222
Mean value	24.0	21.4	2.7	351.7	24.1	21.4	2.8	406.8	21.5	18.9	2.6	386.6

Concerning the statistical relationship between the tumor weight and the spleen weight, the values of "r", "df" and "t" are shown to be 0.44, 133 and 5.72 respectively. Therefore, the correlation between the two may be considered significant. However, in regard to the relationship between the host weight—tumor weight and spleen weight, the values of "r", "df" and "t" are 0.28, 133 and 3.46 respectively. Thus, the correlation between them is not statistically significant. The data point out the fact that the host weight—tumor weight relationship does not necessarily correlate with the body weight.

2) *Splenic change following injection of blood from tumor-bearing mice.* A study was designed to determine the amount of splenic enlargement in normal mice following an intraperitoneal injection of blood from tumor-bearing mice. Also a correlation was sought between the degree of splenic enlargement and the duration following the blood injection. The blood cells collected from the tumor-bearing animals were administered intraperitoneally to the 90 normal mice. The animals were divided into 9 groups, each consisting of 10 mice. Each group was killed at the intervals of 2 days up to 18 days following the first injection of blood. Some groups received 0.5×10^7 red cells while others received 0.1×10^7.

As shown in Figure 2, most mean values of the splenic weights following the injection of blood were higher than those in the untreated animals. However, the relationship between the splenic change and "killed day" was noted to be statistically not significant. The high value of the spleen weight is retained almost during the entire course of the test. It is probable that the value may be reduced gradually after the period noted. Judging from the above result and from the fact that the appearance of ascites will become obvious in about 8 days in those cases in which the tumor cells are mistakenly injected intraperitoneally, the mice were all killed on the 8th day after the injection.

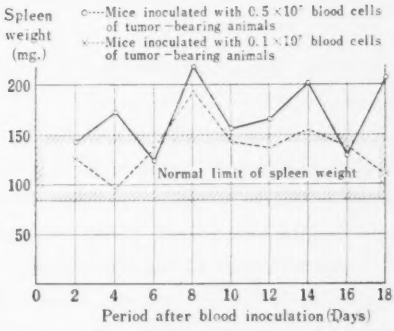

Fig. 2 The relationship between the degree of splenic enlargement and the period after the blood injection of tumor-bearing animals.

Next, the relationship between the number of the injected blood cells and the degree of splenic change was studied. Thirty-nine normal mice were divided into three groups of 13 each. Mice from each group were given intraperitoneal injections of 10^7, 10^6 and 10^5 red cells respectively. The difference of mean values between groups 1 and 2 proved to be significant as shown by the result of t-test shown in Figure 3 and Table 2. Moreover, the difference between groups 1 and group 3 show the result to

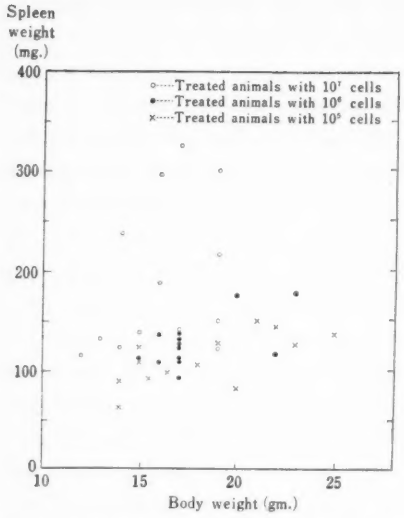

Fig. 3 The relationship between the number of injected blood cells and the degree of the splenic change.

Table 2. The result of the t-test about the difference of the average values.

Source	ss	df	ms	F
(1) The relationship between group 1 and group 2.				
Betw.	21924.0	1	21924.0	6.548 *
W/in.	80352.5	24	3348.0	
Total	102276.5	25		
(2) The relationship between group 1 and group 3.				
Betw.	42161.9	1	42161.9	12.919**
W/in.	78323.2	24	3263.5	
Total	120485.1	25		
(3) The relationship between group 2 and group 3.				
Betw.	3279.4	1	3279.4	4.168 ns
W/in.	18881.2	24	786.72	
Total	22160.6	25		

Note: According to the F-distribution, 6.548 is within the level of reliability of 5 percent and 12.919 is within that of 1 percent.

be similar. On the other hand, there seems to be no special relationship between groups 2 and 3.

3. ***The animals receiving the largest number of blood cells showed the highest values in spleen weight.*** Judging from these results, it seemed that more than 10^7 cells are needed to cause the enlargement of spleen, therefore, more than 10^7 cells were subsequently injected into animals examined later.

In order to compare the above results with the controls, the influence of the blood of untreated animals upon the spleens of normal mice was studied. The blood was pooled from 22 normal mice and separated into blood cells and serum. The number of blood cells injected into the experimental mice was 10^7 and corresponded to the largest amount given in the previous experiment. In addition,

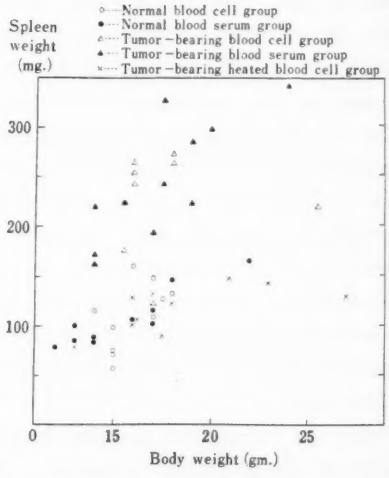

Fig. 4 The spleen weights of normal animals inoculated with blood (cells and serum) of normal mice, and untreated blood (cells and serum) and heated blood (cells and serum) of tumor-bearing animals.

Table 3. The result about the difference of the mean values of spleen weights in the animals treated with various treatments.

	df	F
N-blood cell group× T-unheated blood cell group	19	22.85 **
N-blood serum group× T-unheated blood serum group	19	43.65 **
N-blood cell group× T-heated blood cell group	19	0.784 ns
T-unheated blood cell group× T-heated blood cell group	19	19.16 **

Note: N: Normal animal T: Tumor-bearing animal

the influence of normal pooled sera on the spleen of the normal mice was examined. The volume of pooled sera was adjusted so as to be equivalent to the amount of injected blood cells. The results are shown in Figure 4 and Table 3. From these data it is obvious that neither blood cells nor blood sera of normal mice alters the spleen weight of normal mice.

3) *Some observations on the factor from tumor animals responsible for the splenic enlargement in normal mice.* From the results described above, the presence of a factor in the blood of Ehrlich's carcinoma-bearing mice which caused the splenic enlargement in normal mice was confirmed. An attempt was made to characterize the nature of this factor.

Using the same technique previously described, only blood cells were obtained from the tumor-bearing animals, and the cells were heated at 60°C for 30 minutes. The heat-inactivated blood cells were then diluted with physiological salt solution and injected intraperitoneally into normal mice. The amount of injected blood was adjusted so as to be equivalent to 10^7 cells by adding physiological salt solution. For comparison, untreated blood cells and serum of tumor-bearing animals and blood cells and serum of normal mice were employed as controls in this experiment. The results are shown in Figure 4 and Table 3. As shown clearly from these data, both groups of mice which were given injections of untreated blood cells and untreated blood serum respectively resulted in increased splenic weight. On the other hand, it is obvious that heated blood cells did not cause splenic enlargement as did the normal blood. This heat labile factor, therefore, appears to be present in both blood cells and serum of tumor-bearing animals and its effect is lost by the heat at 60°C for 30 minutes.

As will be described later, the chemical nature of the active factor was not examined precisely in blood samples obtained from the individual mouse. Owing to the fact that the volume of blood which could be obtained from a single mouse was too small, the various tests carried out were limited and detailed analyses were not attempted.

4) *Reconsideration of blood samples from the individual mouse.* All the preced-

ing experiments were carried out on pooled blood samples collected from several tumor-bearing mice. Hence, it was necessary to consider whether the splenic effect exists in the blood obtained from the individual mouse. To answer this question, the following study was made: The normal mice were divided into three groups. The first group consisted of 16 normal mice, the second 32 mice and the third 64 mice. The blood cells and serum from one of 8 tumor-bearing mice was injected intraperitoneally into two normal mice in the first group respectively. Eight days after the treatment, the blood injected animal was sacrificed, and then the spleen was removed and weighed. At the same time, blood sample was taken, and the cells and serum, thus obtained, were employed also for intraperitoneal injection into two normal animals of the second group, respectively. The treatments were repeated for each mouse of the third group. The data obtained are shown in Table 4.

The data show the following: The spleen weights of all animals inoculated with blood of tumor-bearing mice, blood cells or serum, are more or less larger than normal. Moreover, the increase of spleen weight becomes more obscured with repeated serial transfer of blood; thus the spleen weights of the third group show a tendency to approach normal values.

On the other hand, in reference to animals treated with serum of tumor-bearing mice, no conclusion could be drawn due to the small number of experimental animals. However, Figure 5 shows the same trend as the groups inoculated with blood cells despite the fact that prompt recovery to normal was not observed.

5) *DNA analysis of mouse spleen.* The foregoing experiments suggest that mice resulted in the enlargement of spleen following the injection of a factor which appears to be related specifically to the tumor-bearing animals. The response to this factor was reflected in the spleen of the host and was represented by an increase in the weight of the organ. The expression of this magnitude of increase is not sufficient insofar as qualitative and quantative analyses are concerned. One can cite a considerable number of observations reported in the literature indicating

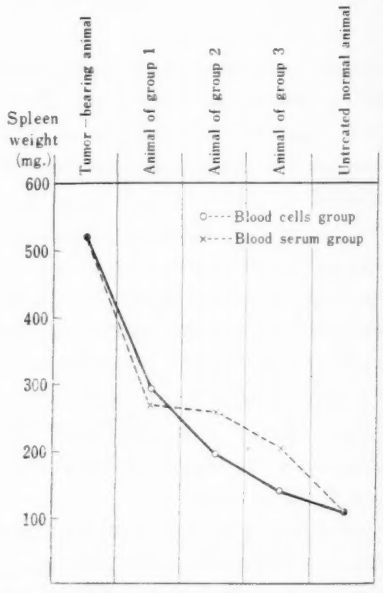

Fig. 5 The change of the mean values of the spleen weights following the repeated inoculation of blood cells and serum of tumor-bearing animals.

Table 4. The data about the each mouse following repeated inoculations of blood of tumor-bearing animals.

Tumor-bearing animals					Group 1: Animals inoculated with blood of tumor-bearing animal.			Group 2: Animals inculated with blood of animal of Group 1.			Group 3: Animals inoculated with blood of animal of Group 2.		
Mouse No.	Host weight (gm)	(Host—Tumor) weight (gm)	Tumor weight (gm)	Splene weight (mg)	Mouse No.	Host weight (gm)	Spleen weight (mg)	Mouse No.	Host weight (gm)	Spleen weight (mg)	Mouse No.	Tumor weight (gm)	Spleen weight (mg)
1	27.0	22.0	5.0	194	1(Bc)	21.0	178	1(Bc)	15.0	164	1(Bc)	23.0	224
					2(Bs)	24.0	184	4(Bs)	22.0	196	8(Bs)	18.5	167
2	30.0	25.0	5.0	631	3(Bc)	16.0	141	5(Bc)	20.0	188	9(Bc)	14.0	71
					4(Bs)	18.0	285	8(Bs)	20.0	300	16(Bs)	19.0	156
3	28.0	24.0	4.0	365	5(Bc)	22.0	447	9(Bc)	20.0	264	17(Bc)	13.0	43
					6(Bs)	22.0	287	12(Bs)	23.0	208	24(Bs)	21.0	106
4	27.0	20.0	7.0	627	7(Bc)	20.0	346	13(Bc)	15.5	203	25(Bc)	10.0	62
					8(Bs)	21.0	244	—	—	—	—	—	—
5	29.0	23.0	6.0	779	9(Bc)	20.0	305	17(Bc)	20.0	348	37(Bc)	19.0	151
					10(Bs)	20.0	363	20(Bs)	22.0	345	40(Bs)	19.0	336
6	23.0	17.0	6.0	387	11(Bc)	20.0	258	21(Bc)	19.0	132	41(Bc)	17.0	270
					12(Bs)	18.0	170	—	—	—	—	—	—
7	18.0	16.0	2.0	520	13(Bc)	22.0	428	—	—	—	—	—	—
					14(Bs)	22.0	220	28(Bs)	19.0	238	56(Bs)	19.0	253
8	23.0	21.0	2.0	540	15(Bc)	20.0	230	29(Bc)	19.0	99	57(Bc)	22.0	164
					16(Bs)	20.0	358	—	—	—	—	—	—

Note: Parts of the data are omitted to avoid complexity.
(Bc) or (Bs) represent the animal inoculated with blood cells or blood serum respectively.

microscopic changes of this organ in tumor-bearing animals, but no definitive conclusions can be drawn from such studies due to the complexity of histological structures of this organ.

Recently, much attention has been paid to the estimation of DNA using histochemical analysis of various organs. Many observers have suggested that the application of this method is more useful than morphologic procedures in many respects. Therefore, a study of splenic DNA content of mice treated under conditions described above was undertaken.

The relationships between the total DNA content per single spleen and the splenic weight of untreated normal mice, tumor-bearing mice and those inoculated with blood of tumor-bearing animals are shown in Figure 6. The total DNA content is plotted against the spleen

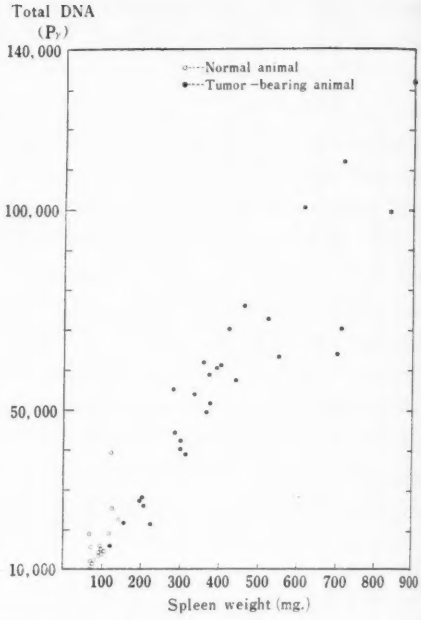

Fig. 6 (a) The total DNA concentrations of the spleens of normal and tumor-bearing animals.

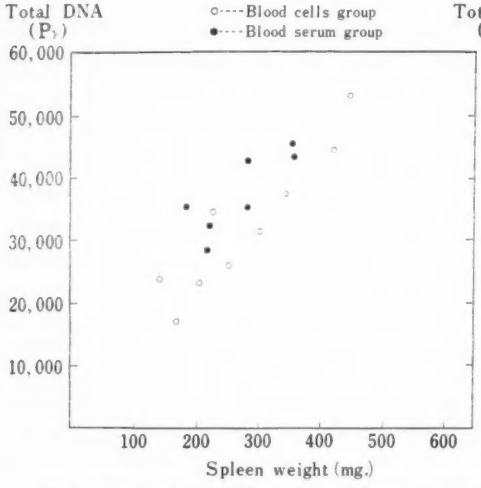

Fig. 6 (b) The total DNA values of the spleens of the animals of group 1.

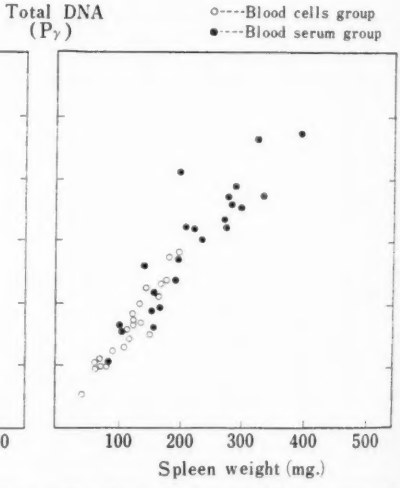

Fig. 6 (c) The total DNA values of the spleens of the animals of group 3.

weight for all animals. The initial impression of the scattergrain is that animals of the four groups show a definite relationship between the spleen weights and their total DNA contents. Thus it appears that irrespective of the kind of treatment given, the total DNA content per spleen seemingly is increased in proportion to the increased weight of the spleen.

The estimations of DNA concentrations per wet and dry splenic weights were made by the method described previously.

Figure 7 shows the DNA concentrations per wet splenic weight in untreated (normal) mice, tumor-bearing animals and those inoculated with blood of normal and tumor-bearing animals. The splenic DNA contents of normal mice has a mean value of 152.87P, with a range of 131.3 to 195.8. The DNA concentrations of this organ in tumor-bearing animals are equal to or less than those of normal spleen. The values in animals injected with the blood of normal animals are almost equal to the control levels. In animals injected with the blood of tumor-bearing animals, the estimation of splenic DNA concentrations, as a whole, is not prominent, but low values in these animals have a tendency to normal after reinjection of the blood. It is interesting that the result is in inverse proportion to the decrease of splenic weight observed by repeating the inoculation of blood of tumor-bearing animals.

Figure 8 shows the DNA concentrations per dry splenic weights in the same

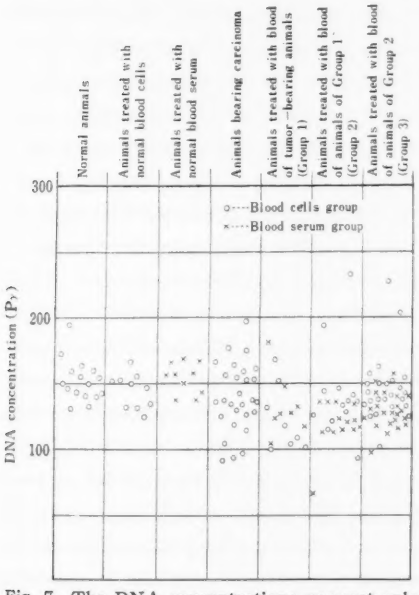
Normal animals
Animals treated with normal blood cells
Animals treated with normal blood serum
Animals bearing carcinoma
Animals treated with blood of tumor-bearing animals (Group 1)
Animals treated with blood of animals of Group 1 (Group 2)
Animals treated with blood of animals of Group 2 (Group 3)
300
200
100
DNA concentration (Pγ)
o----Blood cells group
×----Blood serum group

Fig. 7 The DNA concentrations per wet spleen weights of animals on various concitions.

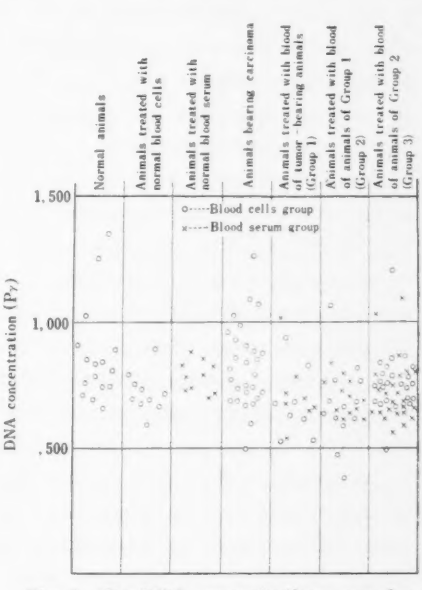
Normal animals
Animals treated with normal blood cells
Animals treated with normal blood serum
Animals bearing carcinoma
Animals treated with blood of tumor-bearing animals (Group 1)
Animals treated with blood of animals of Group 1 (Group 2)
Animals treated with blood of animals of Group 2 (Group 3)
1,500
1,000
,500
DNA concentration (Pγ)
o----Blood cells group
×----Blood serum group

Fig. 8 The DNA concentrations per dry spleen weights of animals on various conditions.

groups of animals treated by procedures described above. In these animals, the DNA values are constant, ranging approximately between 100 and 500, regardless of the kind of treatment given.

Discussion

In determining the changes that occur in the spleen, procedures such as the measurement of size or volume are usable, but in this investigation the method of determining the actual weight of the organ was employed because the method is simple and the error is smaller than by other procedures. In studying the changes of spleen under varying conditions, the primary problem was in determining the mass of the spleen in normal mice. It is generally known that the weight of the spleen of hybrid mice show greater variations than those of inbred strains. Kuroso et al[6] noted that the splenic index of normal inbred mice had a maximum value of 8, and hybrid mice a value of 9-10. He suggested that values greater than these could be used as an "infection-index". These results are generally in agreement with our own findings, but the present report does not employ the unit, splenic index, for the reasons already elaborated.

In 1935, Roffo[1] estimated the values of spleen weight per body weight in groups of normal rats, pregnant rats, rats bearing sarcoma and rats bearing carcinoma; he reported results of 1 : 301, 1 : 105, 1 : 107 and 1 : 190 respectively. Roffo considered the splenic enlargement as being dependent upon hyperplasia of the organ which in turn was associated with cholesterol production in the tumor-bearing animals. He demonstrated that the cholesterol levels in the blood of pregnant and tumor-bearing rats were increased. These results need not always apply to animals bearing all types of experimental tumor, and in certain forms of transplantable tumor such as Takeda's sarcoma[7], the host usually show a remarkable atrophy of this organ. In our own study using Ehrlich's ascites carcinoma, the spleens in tumor-bearing mice were markedly increased in weight, however, the degree of splenic enlargement did not always parallel the stage of neoplastic development.

Roffo noted that when the blood of tumor-bearing rats was injected into normal rats, the spleen became enlarged and the change continued for several days after the inoculation of blood. On the other hand, such a change was not observed when the blood of nontumor-bearing healthy rats were injected into normal animals. Furthermore, he discovered that the spleen weight was increased when blood samples of cancer patients were injected into normal rats. No splenic enlargement occurred when the blood of normal persons was injected. Similar reports are recorded in the clinical literatre. For example, Tsuzuki[8] proposed a method by which he was able to produce splenic enlargement in newts following the injection of urine from cancer patients and Watanabe[9] observed that injections of a substance contained in the

urine of cancer patients caused splenic enlargement in normal rabbits. Recently, in his experiment using Sprague-Dawley rats with Flexner-Jobling carcinoma and Wistar-rats with sarcoma (R-I), Glossley[10] noted that intraperitoneal injections into normal rats of the blood of tumor-bearing rats caused splenic enlargement. The data in the present study confirm the results reported previously by these observers. The fact that the splenic change is not due entirely to the exogenous agents, other than blood components, or to non-bacterial antigens as Rich stated[11], was clarified by tests performed in the present investigation with normal animal blood.

Characterization of the material which is responsible for the splenic enlargement was attempted by Watanabe[9] who examined the substance present in the urine of rabbits that showed splenic enlagement following appropriate injections. He reported that the substance obtained was thermolabile and was inactivated by heating at 70°C for 30 minutes. The material was insoluble in alcohol, ether or acetone, but dissolved in physiologic saline solution. Roffo[1] stated that the splenic enlargement was observed when extracts of rat sarcoma and embryonal tissue or lipoid were injected subcutaneously into normal rats. He further noted that cholestrol in the blood of pregnant and tumor-bearing rats was increased and stated that certain substances, perhaps lipoids contained in the injecta were resorbed selectively by the spleen, and were responsible for the hyperplasia of the organ. This inturn, he believed, promoted the production of tumor-inhibiting or immune. Glossley[10] on the other hand, stated that the cholesterol in the spleen was within normal value. From our own data, it is suggested that the substance may be a protein since it is thermolabile and it may be similar to the substance in the urine described by Watanabe.

An interesting finding by Glossley[10] is the observation that the effect on the spleen did not disappear by serial passage of the substance in rats or by alterating the strain of rats. He assumed that the spleen effect was produced only by the red blood cell fraction. Our observation confirm the effect of serial passage in mice, but do not support the evidence for attributing the splenic change only to the blood cell. In a previous report, the author[12] reported that the spleen effect was not produced by the blood serum, but the present result suggests that the possible explanation for this difference may have been due to inadequate quantity of serum used in the previous study. In understanding Watanabe's and Tsuzuki's views, it is necessary also to assume the presence of active factor in the serum.

Recently, Korinth[13] recognized morphological changes of erythroid cells in cancer patients and believed that these changes might be dependent upon a cancer-toxin. Similarly, Fonti[14] reported other microscopic changes of erythrocytes in cancer patients. Although these observations may not be characteristic of cancer cases, they appear to be due to an inhibitory action of the cancer upon the hematopoietic system, especially on the erythroid cells. What the relationships are between the

morphological changes of red blood cells and the factor affecting the splenic mass should be investigated further.

Numerous studies have appeared in the literature on the subject of nucleic acid contents in organs of various animals. Since Boivin, Vendrely and Vendrely's report[15], it is generelly well recognized that the nucleic acid content of a normal nucleus in the same species is constant, irrespective of the organ or tissue. However, the nucleic acid content in a given mass of tissue is dependent upon the relative proportion of nucleus to cytoplasm for any given cell, therefore, in organs such as spleen and thymus in which the cells are compactly arranged and in which the nucleo-cytoplasmic ratio is relatively great, the amount of nucleic acid is abundant. Schneider[16] reported 144 mg per 100 gm wet tissue of DNA P in the normal mouse spleen, while Shibano[4] gave a DNA value of 92 γ P per 100 mg of wet tissue. The DNA values of mouse spleen expresed as γ P/100 gm wet tissue approximately equal the values cited by these authors.

Cerecedo et al.[17] found an increase in the nucleic acid content of liver, kidney and lung of mice during gestation and those animals bearing sarcoma 180. An increase of DNA concentration of the organs is probably dependent upon the rate of cell division as suggested by Kelly and Jones[18]. Cerecedo, however, stated that no definite conclusion is warranted in respect to changes in the DNA value of spleen and thymus, because the DNA value for these organs in normal animals was extremely variable. It should be pointed out that his date clearly indicate that the DNA value (mg/dry wet tissue mg) of the spleen was markedly decreased during the second week following the inoculation of tumor. In this respect, Cerecedo's result shows good agreement with our previous report which showed that some of the splenic DNA values of mice bearing Ehrlich ascites carcinoma were obviously smaller than those of control mice on 20th days following the inoculation of tumor cells. Our present data show that the DNA concentration (wet tissue 100 mg) of the spleen of tumor-bearing animals are equal to or less than those of controls. It appears from these data that the wide distribution of these values may be a reflection upon the variability of splenic histologic patterns associated with the process of splenic enlargement. For the same specimens, the DNA concentration per mass of dry tissue (100 mg) was essentially within normal values and the total DNA content of the spleen of tumor-bearing animals showed a parallel rise to the increase in total spleen weight.

Takizawa[19] stated that the spleen of rats bearing transplantable hepatoma resulted in a marked increase of spleen weight and volume. He studied the spleen specimens microscopically and found that they show marked proliferation of the splenic pulp, and noted especially prominent myeloid foci which consisted of myeloblasts, variable stages of polymorphonuclear cells and giant cells. The reaction-center of the lymph

follicles was greatly enlarged, but no evidence of proliferation of lymphocytes was demonstrated. Due to the proliferation of the splenic pulp, there was marked narrowing of the sinusoids and the latter were filled with a small amount of red blood cells. From these findings, he concluded that the splenic enlargement was not only due to passive congestion but also was dependent upon a marked increase of myeloid foci and giant cells.

Similarly, Begg[20] reported that the enlarged spleen of tumor-bearing rats has many large clear round cells, megakaryocytes, and reticulum cells in addition to embryonal myelopoiesis. However, as suggested by Gordon et al,[21] the pathogenetic appraisal of the splenic enlargment cannot be adequately demonstrated by simple morphogenetic analysis alone. Therefore, in our investigation, DNA determinations supplemented the histological analysis. As already stated, the splenic tissue is composed of various kinds of cells which possess different nucleocytoplasmic volume ratios, so the DNA contents of such cells probably differ in amount. Theoretically, the estimation should be made on cells of each type, but such procedures are not practical at the present time. Using their excellent method of separating lymphocytes from the spleen, Menten et al[22] reported the nucleic acid contents of lymphocytes in normal and leukemic mice, however, this procedure did not always separate the lymphocytes from other cells. For these reasons, the limitation in the estimates of DNA made in our studies is recognized, but neverthelees, it is believed to be more reliable than measurements by other methods in estimating the actual magnitude of change related to lymphoytes in the spleen.

In tumor-bearing animals, there is a marked increase of total DNA which is in proportion to the enlargement of the spleen, but the DNA content per dry tissue is within normal limits. The constancy of DNA content (dry splenic weight) suggests uniform distribution of the cellular components in this organ for the tumor-bearing animals as well as in the normal mice. It further shows that the proliferation of the cellular elements in the spleen is mainly responsible for the splenic enlargement rather than congestion. In mice inoculated with blood of tumor-bearing animals, the splenic DNA content per wet tissue is decreased in amount and have a tendency to return to normal with repetition of inoculation. This suggests that with inoculation of red cells, the splenic enlargement is due to congestion of the organ, because mature erythroytes have no nuclei, hence no DNA.

From these results, it is concluded that the splenic enlargement observed in tumor-bearing animals is accompanied by active cellular proliferation in the spleen and secondarily by the phenomenon of congestion which experimentally can be shown to result from the inoculation of erythrocytes obtained from tumor-bearing animals.

Summary

The purposes of this experiment are to examine the splenic changes in tumor-bearing animals and to clarify the mechanism and pathogenesis of this phenomenon.

Male mice were employed as the experimental animals and Ehrlich's ascites carcinoma as the transplantable tumor. The results are summarized below:

1) The tumor-bearing mouse showed a pronounced increase in the splenic weight and yet a good correlation between the tumor-weight and the splenic weight was noted.

2) The blood of the tumor-bearing animals is believed to contain a factor which has the property of causing splenic enlargement of the host following its intra-abdominal inoculation and this effect can be observed by serial passage through several normal mice.

3) The factor was contained in the blood serum as well as in the blood cells of tumor-bearing mice.

4) The active material was thermolabile.

5) From the DNA analysis of the spleens of mice treated under various conditions, it was suggestive that the factor in the blood of tumor-bearing animals caused the hyperplasia of cells in the spleen and, secondarily, congestion of the organ. The rate of hyperplasia was shown to prallel the rate of tumor growth.

References

1) Roffo, A.H.: La reacción biológica del bazo en el cáncer provocado por la sangre humana de cancerosos. Bol. Inst. de med. exper, para el setude, y trat. del cancer. **10,** 745-756, 1933 (quoted from Am. J. Canc., **23,** 361, 1935).

2) Murata. Y., Ishida, H., Kono, O. and T. Miyaji: On the relation between number of transplanted tumor cells or size of solid tumor of Ehrlich carcinoma and their growth after transplantation. Gann, **45,** 410-412, 1955.

3) Nabeshima, T.: Studies on the condition of growth and metastasis of Ehrlich ascites carcinoma in mice. Med. J. Osaka University (Japanese edition), **7,** 118-134, 1955.

4) Shibano, S.: Quantitative measurement of the growth of Ehrlich ascites carcinoma and its application to chemotherapy of tumor. Med. J. Osaka University (Japanese edition), **7,** 15-22, 1955.

5) Fiske, C.H. and Y. Subbarow: The colorimetric determination of phosphorus. J. Biol. Chem,. **66,** 375-400, 1925.

6) Kuroso, C. and H. Matsubara: Studies on the splenic weights of normal mouse. Japanese J. Hygiene (Japanese edition), **11,** 303-309, 1956.

7) Takeda, T.: The antigenicity of tumor cells (Japanese edition), Tokyo, Nanzando, 1957.

8) Tsuzuki, T.: On the diagnosis of cancer. Nagasaki Igakkai Zatsushi, (Japanese edition), **22,** 124-139, 1944.

9) Watanabe, M.: Studies on the substance present in the urine of cancer patient which shows splenic enlargement in rabbit following injection. Acta Medica Hokkaidonensia (Japanese

edition), **22**, 878-888, 968-978, 1944.

10) Grossley, M. L.: Spleen enlargement in normal rats caused by blood from rats bearing certain transplanted tumors. Proc. Am. Ass. Canc. Res., **2**, 11, 1955.

11) Rich, A. R.: Acute splenic tumor produced by non-bacterial antigen. Proc. Soc. Expl. Biol. Med., **32**, 1349-1351, 1935.

12) Murata, Y., Ogata, M. and Y. Hashimoto: The influence of the blood of tumor-bearing animals on the spleen of the normal mice. Gann, **47**, 808-810, 1956.

13) Korinth, V. E.: Morphologische Veränderungen des erythropoietischen Systems bei Tumorerkrankungen. Blut, **1**, 161-164, 1955.

14) Fonti, C, J.: Die hämatologische Diagnose des Krebses. Med. Klin., **49**, 1402-1403, 1954.

15) Boivin, A., Vendrely, R. and C. Vendrely: Biochemie de l'hérédité. L'acide désoxyribonucléique du noyau cellulaire, dépositaire des caractére héréditaires; arguments d'order analytique. Compt. Rend. Acad. Soci., **226**, 1061-1063, 1948.

16) Schneider, W. C.: Intracellular distribution of enzymes. I. The distribution of succinic dehydrogenase, cytochrome oxidase, adenosinetriphosphatase and phosphorous compounds in normal rat tissue. J. Biol. Chem., **165**, 585-593, 1946.

17) Cerecedo, L. R., Reddy D. V. N., Piccio, A., Lambardo, M.E. and J.J. Travers: Nucleic acid changes in tumor-bearing mice. Proc. Soci. Expl. Biol. Med., **78**, 683-687, 1951.

18) Kelly, L.S. and H.B. Jones: Effect of neoplastic tissue on the turnover of desoxypentose nucleic acid. Science, **111**, 333-334, 1950.

19) Takizawa, N.: Über die Veränderung der hämatopoietischer Organ der Ratte durch die Transplantation mit dem experimentell erzeugten Lebercarcinoma. Gann, **31**, 665-672, 1937.

20) Begg, R.W.: Problems arising from a study of tumor host relations. In Canadian Cancer Conference. New York, Academic Press, Inc. **1**, 237-253, 1955.

21) Gordon, A. J., Holden, E.C. and S Feitelberg: A quantitative approach to the study of splenomegaly. Arch. path., **46**, 320-337, 1948.

22) Menten, M.L., Willis, M. and W.D. Wright: Nucleic acid content of splenic lymphocytes in normal and leukemic mice. Canc. Res., **13**, 729-732, 1953.

23) Miyaji, T.: The advance of medicine (No. 7), Studies on nucleic acids in tumor. Edited by R. Kinoshita (Japanese edition). Kyoritsu Published Inc. Tokyo, 1951.

24) Chargaff, E. and J. N. Davidson: The nucleic acids. Academic Press Inc., Publishers, New York, N. Y. 1955.

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PRELIMINARY NOTE ON THE INDUCTION OF PULMONARY TUMORS IN MICE BY ISONICOTINIC ACID HYDRAZIDE FEEDING*

(Plate III)

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The investigations leading to the discovery of the induction of pulmonary tumors in mice by feeding isonicotinic acid hydrazide (INH), is briefly reported in this preliminary note. It is possible that this may lead to a reconsideration of the problem of treatment of tuberculosis by applying INH daily for a long period routinely, since the induction of pulmonary tumors in mice through oral administration of INH has now been confirmed by the following results.

Twenty five albino female dd mice, weighing 20g and 2 to 3 month old, were used. Mice were fed *ad libitum* on Oriental diet M** containing a definite amount of INH. Since a small amount of olive oil was added to the mixture, INH adhered well to the powdered diet and there was little danger of mice not taking INH with the diet. The initial amount of INH added to the diet was 3g per kg of the diet. The animals showed substantial decrease in body weight within one or two weeks. Then the amount of INH was decreased to one half until the body weight of mice recovered to the initial. Three months later, the amount of INH was increased again to 3g per kg. The body weight of animals was measured every week. The average amount of food intake per mouse per day, was about 1.5g, from which it was assumed that the amount of INH intake per mouse per day was 1.5–3mg. This dose is equivalent to 10 to 15 times the amount applied to patients. Green vegetables were given to the mice from time to time and water *ad libitum*.

By the above experiment, 10 of mice died of intercurrent infections or convulsions during the first three months. The final effective number consisted of 15 mice, or 60 per cent. The first pulmonary tumor was observed in a mouse sacrificed on the 134 th day. The nodules measuring up to less than 1 mm in diameter were semi-transparent and slightly elevated. These were proved to be adenomas upon microscopical examination. All other mice were sacrificed since that date and the majority of them showed more or less adenoma or adenomatous growth of lung as may be

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** Oriental diet M consists of protein 24.8%, fat 5.6%, ash 5.7%, carbohydrate 51.4% and moisture 7.0%.

Table

Mouse No.	Period of survival (Days)	INH Ingested (mg)	Body weight (g)	Liver weight (g)	Spleen weight (mg)	Lung weight (mg)	Lung Findings*
1	134	222	17	0.8	94	135	+
2	〃	〃	17	1.0	68	130	++
3	158	286	18	0.8	120	140	+
4	〃	〃	17	0.8	188	158	++
5	174	329	15	0.9	90	121	−
6	〃	〃	18.5	1.0	109	146	+
7	〃	〃	19	1.2	114	161	++
8	〃	〃	16	0.8	64	118	±
9	〃	〃	21.5	1.1	87	151	++**
10	〃	〃	17.5	0.9	98	197	−
11	210	426	18	1.2	85	169	++**
12	〃	〃	21	1.0	168	180	++
13	〃	〃	19	1.0	120	198	++
14	〃	〃	20	1.1	194	178	++
15	〃	〃	20	1.0	126	160	++

*Lung findings: −: normal, ±: hypercellularity and disorientation of cell relationships, +: hyperplastic foci and ++: pulmonary tumor.

**Pulmonary tumors were detected microscopically.

seen in the table.

In expressing the nature of the lung findings in the table, we adopted the use of double plus ++, plus +, plus-minus ± and minus - signs, indicating pulmonary tumor, hyperplastic foci or cell aggregations, diffuse hyperplasia and normal, respectively. In all cases, these diagnoses were confirmed by microscopical examination.

The surface of the tumor was greyish white in color with pearly glistening. It was fairly well demarcated from the surrounding tissue and protruded from the pleural surface. The foci were multiple. Microscopically, the tumor cells were cuboidal or columnar, similar in size, and the nuclei were round, oval and contained abundant chromatin. They were located in about the middle of the cells. The mitotic figures were not numerous. The cells were, in general, arranged in a single layer, and sometimes formed the glandular lumina in a papillary fashion or were relatively solid in appearance (Figs. 1 and 2). The stroma was scanty and consisted of a few fibrocytes. Their growth was expansive. These growths are identical in structure with those described as primary tumors of the lung, or induced tumors by carcinogenic hydrocarbons, urethane or nitrogen mustard by many previous investigators (1, 2, 3, 4).

Earlier changes apparently began as the general increase in cellularity and

disorientation of cell relationships (Fig. 3). There were relatively larger and more definite areas of cellular accumulation. These areas were encountered most frequently in subpleural and perivascular sites. They consisted mainly of cells that were not distinguishable from the cells seen in the alveolar walls of normal lungs (Fig. 4). These hypercellular areas are designated as hyperplastic foci, as described by the previous investigators (5, 6). Distinction between hyperplastic foci and pulmonary tumor was possible on the basis of the adenomatous pattern of tumor and of the larger cells comprising the tumors. Some evidences of proliferative activity of bronchiolar epithelium also were found not infrequently. As to their point of origin, the authors consider it to be from the alveolar epithelium at present.

The rate of the spontaneous incidence of pulmonary tumors in dd mice has not been determined, but in this laboratory, microscopical examinations on 60 control dd mice from 1 or $1^1/_2$ year old from the same breeding stock have found 9 isolated pulmonary tumors (15 %).

Since the discovery of INH by Fox(7), many attempts were made to elucidate the pharmacodynamic actions of the chemical. Recently, Juhàsz, Baló and Kendrey have reported that the carcinogenic action in mice was due to the intraperitoneal injections of the chemical(8). However, the appearance of pulmonary tumors following the ingestion of INH-containing diet may be of significance, for the chemical is very extensively used in the chemotherapy against tuberculosis at present.

In another experiment the amount of INH was increased to 5g per kg of diet. This greatly shortened the time required for the induction of pulmonary tumors(100 days). Similar hyperplastic changes can be induced by INH feeding also in rats and other animals. Further experiments on the induction are now under way in this laboratory, and the results will be reported elsewhere in full when experiments are completed.

Conclusions

Pulmonary tumors induced in dd mice by the feeding of isonicotinic acid hydrazide begin to appear 134 days after the beginning of the experiment. Since the application of this kind of chemicals is very prevalent in treating tuberculosis, it may be necessary to reconsider its use from the point of its carcinogenic action.

Explanation of Figures (Plate III)

Fig. 1. Pulmonary tumor induced by INH feeding. Mouse No. 3.
Fig. 2. Higher magnification of Fig. 1.
Fig. 3. Area from generalized hypercellularity and disorientation.
Fig. 4. Area from hyperplastic focus.

LITERATURE

1) Murphy, J.B., and Sturm, E.: Preliminary lung tumors in mice following the cutaneous application of coal tar. J. Exper. Med. 42, 693-700 (1925).

2) Grady, H.G., and Stewart, H.L.: Histogenesis of induced pulmonary tumors in strain A mice. Amer. J. Path. 16, 417-432 (1940).

3) Nettleship, A., and Henshaw, P.S.: Induction of pulmonary tumors in mice with ethylcarbamate (urethane). J. Nat. Cancer Inst., 4, 309-319 (1943).

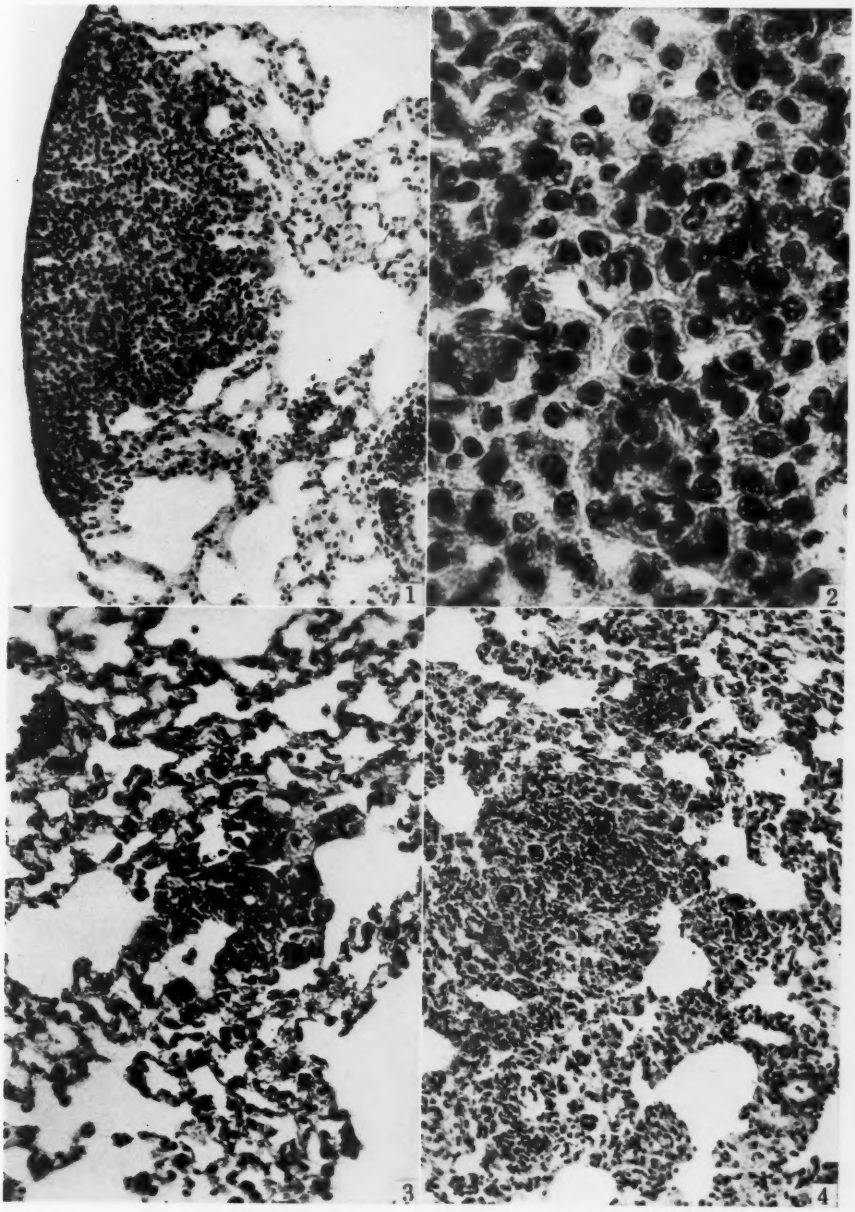
4) Heston, W.E.: Induction of pulmonary tumors in strain A mice with methylbis (β-chloroethyl) amine hydrochloride. J. Nat. Cancer Inst., 10, 125-130 (1949).

5) Mostofi, F.K., and Larsen, C.D.: The histopathogenesis of pulmonary tumors induced in strain A mice by urethane. J. Nat. Cancer Inst., 11, 1182-1221 (1951).

6) Shimkin, M.B.: Pulmonary tumors in experimental animals. Ad. Cancer Res. 3, 223-267 (1955).

7) Fox, H.H.: Synthetic tuberculostatus. III. Isonicotinaldehyde thiosemicarbazine and some related compounds. J. Org. Chem., 17, 555-562 (1952).

8) Juhâsz, J., Baló, J., and Kendrey, G.: Über die geschwulsterzeugende Wirkung des Isonicotinsäurehydrazid (INH), Z. Krebsforsch., 62, 188-196 (1957).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